Characterisation of parsnip canker pathogens and identification of plant resistance

Lauren Harriet Kate Chappell

A thesis submitted in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

The University of Warwick, School of Life Sciences

September 2016

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
bp	Base Pair
C	Celsius
CDA	Czapek Dox agar
CDH	Cellobiose dehydrogenase
CFU	Colony forming unit
cm	Centimetre
cM	Centimorgan
d	Day
d.f.	Degrees of freedom
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EF1-α	Translation elongation factor
EtOH	Ethanol
F	Forward
g	Gram
GBS	Genotype by sequencing
GLM	Generalised linear model
h	Hour
ha	Hectare
ITS	Internal transcribed spacer region
KASPar	Kompetative allele specific PCR
Kb	Kilo base
L	Litre
LOD	Logarithm of the odds
LOG	Logarithm
LSD	Least significant difference
LSU	Large subunit
m	Metre
M	Molar mass
MA	Malt agar
MAS	Marker assisted selection
MAPK	Mitogen-activated protein kinases
mg	Milligram
min	Minute
ml	Millilitre
MQM	Multiple QTL mapping
ng	Nanogram
NGS	Next generation sequencing
nm	Nanometre

PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PKA	Protein Kinase A
pM	Picomole
ppm	Parts per million
QTL	Quantitative trait loci
R	Reverse
rDNA	Ribosomal deoxyribonucleic acid
REML	Restricted or residual maximum likelihood
RNA	Ribonucleic acid
RO	Reverse osmosis
RpbII	RNA polymerase
rRNA	Ribosomal ribonucleic acid
s	Second
SEM	Standard error of the mean
SNA	Synthetic nutrient-poor agar
SNP	Single nucleotide polymorphism
sp.	Species
spp.	Species
SSU	Small subunit
STR	Sugar transporter
tMT	tRNA methyl transferase
tRNA	transfer ribonucleic acid
TTF	Triosephosphate transporter family
TUB2	Beta-tubulin
UK	United Kingdom
v/v	Volume by volume
w/w	Weight by weight
µl	Microlitre
µmol	Micromole

Acknowledgements

Firstly, I would like to express my gratitude to Dr. Guy Barker and Dr. John Clarkson for their guidance, encouragement, and support throughout the course of my PhD. Without their advice and thoughtful comments this thesis would have not been possible.

I would like to thank Dr. Graham Teakle for his expertise and patience, and to Sue Kennedy, Dr. Adrian Dunford, Dr. Richard Tudor and everyone at Elsoms Seeds Ltd who helped make the phenotyping experiments such as success. I would also like to express my gratitude to all members of the Clarkson group for their help, especially Claire Handy, for sharing her knowledge and passion of plant pathology. Further thanks must go to Spencer Collins and Lorenzo Pellis for all their support and advice on the statistical analysis and modelling of my data, and to the PhD students and research staff based at Wellesbourne, for their help and friendship over the last four years.

I acknowledge the financial support from the BBSRC and Elsoms Seeds Ltd, and the opportunities they gave me to gain experience of the agricultural industry.

Finally, a huge thank you must go to my Mum, for always being there for me, and for always believing in me. Without your unconditional love and support, I would have never had the courage to make it this far.

Declaration

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by the author except where otherwise stated.

Summary

Parsnips (*Pastinaca sativa*) are a speciality crop, covering 3000 hectares across the UK, with a 93,000-tonne production and economic value of greater than £31M annually. Currently, the major constraints to production are losses associated with root canker disease, caused by a range of fungal pathogens. With no specific fungicides, development of long-term, sustainable resistance to parsnip canker is highly desirable. This work characterises the pathogens responsible, and develops tools to facilitate breeding for quantitative resistance to root canker diseases.

Isolations and molecular characterisation of pathogens responsible for parsnip canker highlighted a range of fungal species, whilst canker symptoms were found to be clearly associated with certain pathogens. *Cylindrocarpon destructans*, *Mycocentrospora acerina* and to a lesser extent *Itersonilia pastinacae* were identified as the primary pathogens responsible for causing parsnip canker in the UK.

Itersonilia spp. isolates from a range of hosts were found to infect parsnip roots and leaves, and produce both chlamydospores and ballistospores at a range of temperatures; furthermore, molecular characterisation failed to differentiate between species. For these reasons, Itersonilia should be described as a single species.

For both *C. destructans* and *M. acerina*, isolates showed minimal variation in pathogenicity on parsnip roots and seedlings, and exhibited mycelial growth even at low temperatures. Phylogenetic analysis identified a species complex for both pathogens that could not be resolved by the *ITS* (Internal transcribed spacer) alone.

Finally, parsnip root and seedling assays were developed to determine resistance *to I. pastinacae*, *M. acerina* and *C. destructans* within parsnip populations. QTL analysis of a parsnip genotyping population identified a significant QTL conferring resistance to *M. acerina* for use in a marker assisted breeding programme.

The understanding of the pathology gained in this project will facilitate selection of resistant varieties, benefitting breeders, growers and through reduction in control mechanisms, society in general.

1. General introduction

1.1 *Pastinaca sativa*

1.1.1 Parsnip history

Parsnip (*Pastinaca sativa*) has been grown as a root crop for centuries, both for human consumption and livestock feed (Grieve, 1993). It has also been found to have potential antimicrobial effects against common food spoilage microorganisms including *Escherichia coli*, *Salmonella enteritidis* and *Staphylococcus aureus* (Matejic *et al.*, 2014). Today the parsnip still maintains a niche position within the vegetable market with demand growing year on year (McPherson, 2013). Many modern varieties can trace wild relatives back 10,000 years through the breeding of cultivars with wild parsnip species (Jogesh *et al.*, 2015), but modern agricultural practises have led to dependence upon a small selection of domesticated species (Grandillo *et al.*, 2007).

Parsnips (*Pastinaca sativa*) are part of the Apiacae family, a predominantly aromatic group consisting primarily of herbs (Ostertag *et al.*, 2002; Dirmenci, 2008), and other economically important crops including carrot (*Daucus carota* L.) and celery (*Apium graveolens* L.). A native of Eurasia between the western Mediterranean regions and the Caucasus Mountains (Rubatzky *et al.*, 1999), the genus contains 14 species spread throughout this temperate region (Mabberley, 1989). Evidence of their use as a food is documented as far back as the Romans and Greeks (Hendrick, 1919). Upon the collapse of the Roman Empire many vegetables were lost from agriculture, and the Parsnip was not widely grown again until the Renaissance era in the mid 13th Century (Laws, 2004; Surhone *et al.*, 2010). The fuller fleshy forms we are more familiar with were developed during the Middle-Ages (Trager, 1970; McGee, 1984) after which the species was taken to the New World by the pilgrims, and subsequently introduced to the

Caribbean, (Simmonds, 1967), Venezuela and Peru by the early 1600s (Cain *et al.*, 2010). The cultivation of sugar beet as a source of sugar, and the introduction of the potato as a new source of starch in the early 1600s (Hawkes & Franciso-Ortega, 1993), saw the parsnip gradually decrease in popularity (Cain *et al.*, 2010). Following the global spread of the parsnip, it was naturalised from cultivation in many countries and reverted to a phenotype similar to that of the wild-type species. Two varieties var. *sativa* and var. *pratensis* are now frequently regarded as noxious weeds (Kartesz, 1994) due to the terpenes and furanocoumarins within the sap that react with the sunlight when in contact with the skin. This phytophotodermatitis reaction produces blisters, a rash and occasionally a permanent scaring of the tissue in a similar manner to other umbelliferous crops such as celery and parsley (*Petroselinium crispum*) (Engelberth, 2006). This has led to some countries actively controlling wild parsnip populations to reduce the establishment of new plants (Cain *et al.*, 2010).

1.1.2 Parsnip physiology, growth and seed production

Parsnips are a biennial crop, but can behave as a monocarpic perennial (Averill & DiTommaso, 2007). Plants produce a thick, white/yellow, funnel shaped taproot that can grow to a depth of 1.5 m under suitable conditions (Gleason & Cronquist, 1991). With age the taproot may become branched, wiry and tough (Cain *et al.*, 2010); the flavour and quality of the root may also decrease (Shattuck *et al.*, 1989). Rosettes grow close to the soil surface and develop alternate, pinnately compound leaves that can grow up to 15 cm in length (Averill & DiTommaso, 2007). Flowering stems are hollow with sparse hairs, growing up to a height of 40-200 cm with alternate broad leaves (Gleason & Cronquist, 1991). Inflorescences are large, compound, determinate umbels approximately 10-20 cm wide (Cain *et al.*, 2010). Petals are yellow, with small or no sepals; fruits contain two flattened mericarps each with one seed (Hendrix *et al.*, 1991). These seeds are usually straw-coloured to light brown with the

terminal umbel generally producing larger seeds than the lateral umbels (Cain *et al.*, 2010; Hendrix *et al.*, 1991). Parsnip germination typically takes between four to eight weeks in the field, significantly longer than most other crops, with percentage emergence also typically lower than equivalent crops. Hendrix (1984) also found that smaller seeds, those from lower umbels or plants of a smaller size, germinated more quickly than larger, heavier seeds. Once seedlings are established, true leaves develop during the summer via a period of rapid growth. Vegetative growth dies-off over winter, leaving only the vegetative root. It is during this period, or the following spring, that the parsnip crop would be harvested (Hendrix, 1984). Parsnip seed production is generally facilitated by the use of bees to ensure thorough cross-pollination. Seed-set typically occurs during the summer months, after a period of vernalisation the previous winter, where several hundred seeds can be collected from a single umbel. Seed quality reduces very quickly; as such, parsnip seed is only viable for one year, meaning growers are required to purchase new seed every season (Hendrix *et al.*, 1991).

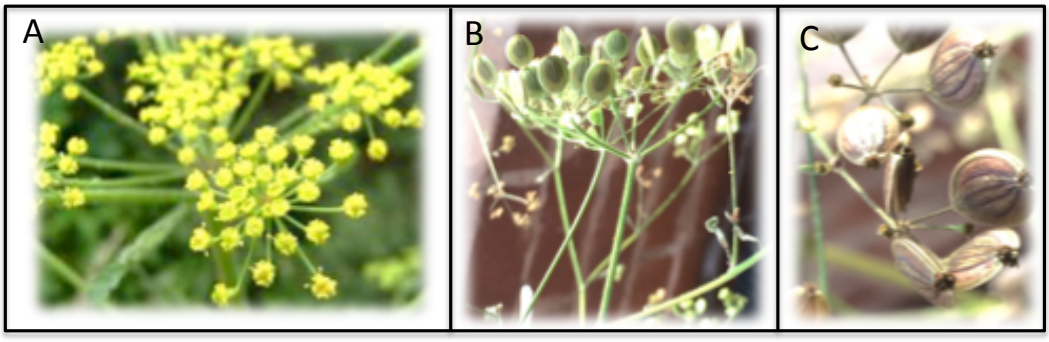


Figure 1.1 – Parsnip development (A) Developing yellow umbel inflorescence of parsnip, flowers radiate from a single point. (B) Fertilised parsnip flowers producing fruit/seed (mericarp). (C) Parsnip seed, characterised by two flattened light brown mericarps.

Within the UK, large-scale production of parsnips can mostly be found in Lincolnshire, Yorkshire, Norfolk, Nottinghamshire and Scotland (Surhone *et al.*, 2010). A relative of the carrot (*Daucaus carota*), parsnips are similar in shape, but paler with a stronger flavour and are typically longer and heavier (Surhone *et al.*, 2010). Parsnips are a specialty crop within the UK, due largely to the sharp frost

that is required to develop their unique flavour and size. Roots accumulate sugars including sucrose, glucose and fructose more rapidly at temperatures close to 0°C. As such, roots for human consumption are rarely grown in warm climates, where crop production is instead generally aimed at livestock feed (Shattuck *et al.*, 1989; personal communication). The roots typically grow best in sandy, loamy soil that exhibits good drainage (Surhone *et al.*, 2010); clay, rocky soil increases the chance of fanged, short roots unsuitable for the consumer market. Sowing usually occurs in spring, and harvesting can begin as early as autumn continuing right through to the following spring (Surhone *et al.*, 2010). Approximately 4000 ha of this umbelliferous root crop are cultivated across the UK, with an estimated economic value of £64M annually.

1.1.3 Parsnip pests and diseases

Parsnips are susceptible to a range of pests and diseases, although the high production of defensive compounds such as furanocoumarins and terpenes within the parsnip is thought to reduce the degree to which the crop is affected by various organisms (Cain *et al.*, 2010). Despite this, the greatest cause of crop loss and economic damage is due to fungal pathogens, the causal agents of root canker disease. Amongst those traditionally implicated in causing parsnip canker are *Itersonilia pastinacae*, *Phoma complanata* (Tode) Desm., *Cylindrocarpon destructans*, *Mycocentrospora acerina*, *Alternaria sp*., *Botrytis cinerea* Pers., *Cladosporium cladosporioides*, *Fusarium sp*., *Epicoccum nigrum*, *Pythium sp*., *Rhizoctonia sp*. and *Sclerotinina sclerotiorum* (Cain et al., 2010; Channon 1963, 1965, 1969). In addition to fungal pathogens a range of other pests have also been implicated in parsnip crop losses. Amongst the most destructive insects for growers are the carrot root fly (*Chamaepsila rosae*), a dipteran fly larvae feeding on the taproot causing small brown tunnels on the outer skin of mature roots (Wheatley & Freeman, 1982), and the parsnip webworm (*Depressaria pastinacella*), whose larvae feed off foliage and developing inflorescences. Leafminers, leafhoppers, aphids, and various lepidopterae have also been

reported as pests of cultivated parsnips (Cain *et al.*, 2010). In addition a number of nematodes and viruses have also been implicated in damage to parsnip crops (Conners, 1967; Hicks *et al.*, 1986). Three of the fungi mentioned above, *Itersonilia pastinacae*, *Mycocentrospora acerina* and *Cylindrocarpon destructans* are the focus of this thesis and are described in more detail below.

1.2 *Itersonilia* spp.

1.2.1 *Itersonilia* taxonomy

Itersonilia spp. are a basidiomycetes from the order Cystofilobasidiales, a group consisting primarily of yeasts from cold climates (Millanes *et al.*, 2011). The genus was originally described by Derx (1948) and comprised of only one species, *I. perplexans* Derx., isolated from an infected leaf of Hollyhock (*Alcea rosea*). Later Nyland (1949) added a second species, *I. pyriformans*, but these two species were soon considered conspecific (Olive, 1952; Tubaki, 1952; Sowell & Korf, 1960). The final species, *I. pastinacae,* was described by Channon (1963) after its isolation from diseased parsnip tissue. However, because of 'interspecific' mating reactions the existence of three separate species is strongly questioned (Boekhout, 1991). The key difference between *I. perplexans* and *I. pastinacae* was the presence of thick walled chlamydospores only within *I. pastinacae* isolates (Channon, 1963). However, Boekhout (1991) noted that strains isolated from Chrysanthemum, Parsnip, Anemone and Dahlia all contained Chlyamdospores, indicating this was not an adequate method for differentiating between *Itersonilia* strains.

Itersonilia spp. were identified as the primary cause of black canker within parsnip crops (Channon, 1963); it is additionally known to cause a range of symptoms on a variety of other plant species, including but not limited to: other members of the Apiacae family such as Dill (*Anethum graveolans*: McGovern *et al.*, 2006), Parsley (*Petroselinum crispum*), Coriander (*Coriandrum sativum*) and

Cumin (*Cuminum cyminum*: Lambourne, 2011), as well as members of the Asteracae family including Chrysanthemum (*Chrysanthemum indicum* L.: Gandy, 1996), Sunflower (*Helianthus annus*: McGovern *et al.*, 2006) and edible Burdock (*Arctium lappa* L.: Horita & Yasuko, 2002).

Itersonilia spp. are dimorphic fungi with a fungal and yeast phase of growth (Ingold, 1983). It is distinguished by the presence of mycelium with ballistospores and chlamydospores. The fungal stage is characterised by abundant white, low aerial mycelium when grown on 2 % malt agar (Ingold, 1983). There are clear clamp-connections at the septa, with basal clamp sporogenous cells at the end of lateral submerged mycelial branches (Olive, 1952). When the hyphae break through the surface of the agar, the tip narrows to form a speculum from which the aerial ballistospore develops (Ingold, 1983). Once a sporogenous cell has become exhausted, the hypha below will immediately produce another, which may be repeated several times leading to the formation of a cluster of sporogenous cells. Ballistospores are discharged by the formation of a water drop at the hilar appendix of the spore whilst perched on the sterigma. Contact between the water drop and spore wall leads to wetting of the spore wall resulting in a redistribution of the centre of mass of the spore, this redistribution of mass causes the separation of the spore from the sterigma. Once separated from the sterigma, the water spreads over the entire spore wall resulting in a negative charge across the spore wall, and this electrical repulsion between the spore and sterigma causes the spore to be propelled (Webster *et al.*, 1984; Pringle *et al.*, 2005). Itersonilia also produces chlamydospores, described as thick-walled resting spores. These subspherical, smooth bodies occur within the agar, either in intercalary or terminal position (Ingold, 1986). Chlmaydospores usually occur singularly, or in rows of 2-4 leading to a chain of spores, the oldest appearing first (Ingold, 1986). These cells have a thickened cell wall, and whilst in dikaryophase form a clamp connection that is lacking in monokaryophase chlamydospores (Sowell & Korf, 1960). It has also been noted that isolates

sometimes display a change in pigmentation in relation to differing numbers of chlamydospores (Ingold, 1986; Sowell & Korf, 1960).

In older cultures, sporogenous cells, instead of producing a ballistospore, may give rise to a pear-shaped conidium. This conidium is readily detachable either on the surface or within the agar and increases through budding, resulting in yeast-like developments within the mycelial colony (Ingold, 1983). These yeast-like colonies have a slower growth rate (0.2 mm / day at 20°C) than mycelial colonies (2 mm / day at 20°C), and have never been seen to revert back to a mycelial form (Ingold, 1986).

1.2.2 *Itersonilia* epidemiology

Itersonilia is a seed borne pathogen, with a distinct foliar cycle. It is the cause of seedling blight, leaf spots and necrosis, in addition to root cankers (McGovern *et al.*, 2006; Channon, 1963; Horita & Yasuko, 2002). The foliar transmission is dependent on the production of ballistospores, which are forcibly discharged from the surface of the seed to the surrounding foliage, where they land and form lesions. These lesions in turn develop ballistospores, which are discharged to land on, and infect, new seedlings. As the seedlings mature and develop, the ballistospores coat the newly formed seed completing the cycle of foliar transmission. The transmission of Itersonilia to the parsnip root is not fully understood, but current thought suggests necrotic lesions from the infected foliage drop onto the exposed crown and shoulder of the developing roots in the soil, leading to the development of lesions (Channon, 1969; Gladders, 1997).

1.2.3 *Itersonilia* symptoms

Itersonilia is responsible for a range of symptoms on a number of plant species, including seedling/leaf blight, leaf spots and root necrosis (Lambourne, 2011) on umbelliferous herbs, members of the asteracae family and parsnip, respectively. Identified as the cause of leaf blight on herbs such as Dill (*Anethum graveolens*),

and Fennel (*Foeniculum vulgare*), Itersonilia is responsible for leaf blight symptoms causing foliage to turn brown/yellow through necrosis. Leaf blight symptoms become increasingly worse with wet weather. Growers noting high volume crops such as Parsley suffer from greater crop losses during cooler, wetter weather (Lambourne, 2011).

Itersonilia was identified as being the primary cause of parsnip canker, through isolations from diseased tissue of infected parsnip roots (Channon, 1965). The root lesions associated with *I. pastinacae* have a dark brown/black colouration and appear on the crown and shoulder of the root during the autumn and winter (Channon, 1963). Surface tissues appear ruptured and underlying parenchyma tissue may appear purple in colour (Channon, 1965). The leaf symptoms of necrotic lesions appear as areas of brown tissue surrounded by a green/yellow halo. The brown necrotic tissue eventually falls away leaving an exposed hole in the leaf (Channon, 1963b).

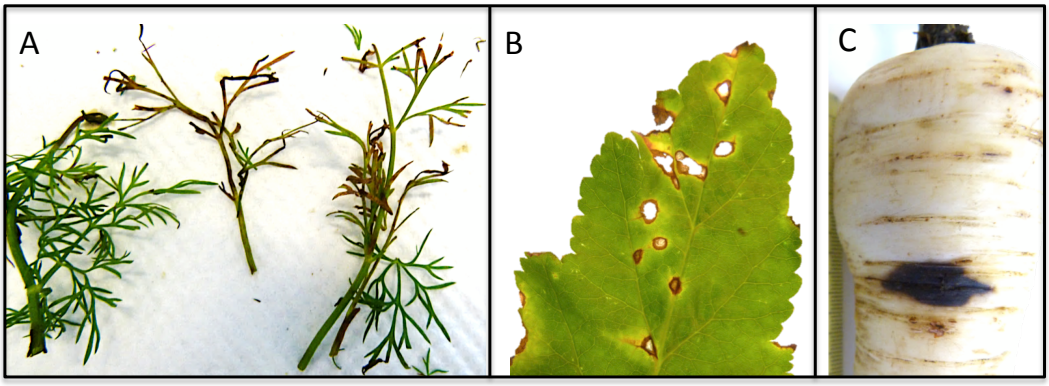


Figure 1.2 – *Itersonilia* sp. symptoms. (A) Dill infected with *Itersonilia sp.* foliar symptoms include brown/yellow leaf blight. (B) Parsnip leaf infected with *Itersonilia* sp., foliar symptoms include brown necrotic lesions surrounded by a green/yellow halo. Necrotic areas eventually fall away leaving an exposed hole in the leaf. (C) Parsnip root infected with *Itersonilia* sp. symptoms include black/brown canker generally forming on the shoulder/crown of the root.

1.3 *Mycocentrospora acerina*

1.3.1 *Mycocentrospora* taxonomy

Mycocentrospora acerina (Hartig.) Deighton is a soil-borne, polyphageous ascomycete from the order Capnodiales (Hermansen, 1992), a group consisting primarily of sooty moulds but also lichens and plant pathogens (Crous *et al.*, 2009). There are multiple synonyms of *M. acerina*, which has led to confusion when describing this plant pathogen (Sutton & Gibson, 1973; Table 1.1).

Table 1.1 – List of synonyms of *M. acerina* (Evenhuis, 1998).

Synonym	Date Renamed
Cercospora acerina Hartig	1880
Sporidesmium acerinum (Hart.) Frank	1896
Cercosporella acerina (Hart.) Arnaud	1918
Cercospora macrospora Osterwalder	1924
Cercospora cari Westerdijk & Van Luik	1924
Cercospora praegrandis Sprague	1937
Centrospora ohlsenii Neergaard	1942
Centrospora macrospora (Osterw.) Neergaard	1983
Ansatospora macrospora (Osterw.) Newhall	1944
Ansatospora acerina (Hart.) Hansen & Tompkins	1945
Centrospora acerina (Hart.) Newhall	1946
Mycocentrospora acerina (Hart.) Deighton	1972

M.acerina is prevalent in temperate areas of Europe, North America, New Zealand and Australia (Neergaard & Newhall, 1951). It is known to affect up to 90 plant species including ornamentals, weed species such as field and wild pansy (*Viola arvensis, Viola tricolor*), cleavers (*Galium aparine*), creeping buttercup (*Ranunculus repens*), hedge nettle (*Stachys palustris*) (Hermansen, 1992), and a

number of economically important crops. Amongst those of economic importance are lettuce (*Lactuca sativa*) and multiple Umbelliferous crops including carrot (*Daucus carota* L.), carroway (*Carum carvi*), celery (*Apium graveolens*) and parsnip (*P. sativa*).

M. acerina has been noted to have no sexual phase, and isolates from different crops are morphologically identical (Neergaard & Newhall, 1951; Iqbal & Webster, 1969). In culture the mycelium is superficial and immersed, septate, branched and hyaline sized between 4-8 µm; chlamydospores are present, and are usually brown in colour with a diameter of 17-30 µm; usually occurring in chains of between 8-15 cells. *M. acerina* is also characterised by producing large septate condidia of 150-200 µm long and up to 8-15 µm thick along the widest portion (Sutton & Gibson, 1977). Chlamydospores are long-lived survival structures, with evidence they can survive at least two years (Evenhuis *et al.*, 1995).

1.3.2 *Mycocentrospora* epidemiology

Mycocentrospora is known primarily as a storage disease, causing liquorice root rot on carrots and celery. Relatively little is known about the survival and development of the fungus (Wall & Lewis, 1980; Hermansen, 1992) but soil is considered to be the main source of *M. acerina* inoculum. Wall & Lewis (1980) suggested chlamydospores and pigmented mycelium surviving in naturally infested soils are causing transmission of *M. acerina* due to the high positive correlation between levels of chlamydospores in the soil at the time of planting and subsequent infection upon harvest (Wall & Lewis, 1980). Current thought is that chlamydospores and mycelial fragments within the soil attach to the root and crown of carrots and celery leading to the transmission of infection (Hermansen *et al.*, 1997). As *M. acerina* is unable to grow through soil except in the host, rhizosphere proximity of seedlings to clumps of chlamydospores is likely to be the primary means of host contact. This is thought to be even greater

if root wounding is present (Davies et al. 1981). However, passive forms of dispersal via chlamydospore spread when 'earthing-up' during the growing season, and splash dispersal from conidia are also possible sources of transmission (Wall & Lewis, 1979). There is also potential for *M. acerina* to be transmitted through infected seed as was originally suggested by Westerdijk & Van Luijk (1924) in relation to caraway seeds. This was proven by Gill (1971) on wild pansy (*Viola tricolor L.*) and further proven by Evenhuis (1995) on caraway.

1.3.3 *Mycocentrospora* symptoms

The symptoms caused by *M. acerina* on parsnips are very similar to those caused by Itersonilia. The periderm of the crown/shoulder of the root appears unbroken with purplish-black diseased tissue. As the infection progresses the subsurface tissue becomes exposed and the lesion may appear to be surrounded by a brown-red band (Channon, 1965).

Figure 1.3 – *Mycocentrospora acerina* symptoms. (A) *M. acerina* infected wild pansy (*Viola tricolor*); foliar symptoms include black lesions (Royal Horticultural Society, 2016). (B) Carrot infected with *M. acerina*, symptoms include black/brown sunken lesions. (C) *M. acerina* infected parsnip root, symptoms include dark brown/black sunken lesions.

Liquorice root rot symptoms on carrot and celery have very similar symptoms to parsnip, being characterised by black-brown sunken lesions that penetrate deep into the root. On carrot lesions generally appear on the crown/shoulder of the root, but may develop anywhere along the taproot. In celery the elongated black

lesions develop at the base of the petioles and are commonly referred to as crown rot (Koike *et al.*, 2007). Within wild hosts, *M. acerina* is more distinguishable by foliar symptoms including black, irregular lesions on the leaves, and petioles. In the case of wild and field pansy (*Viola arvensis, Viola tricolor*) lesions were also commonly found on the inflorescence (Hermansen, 1992).

1.4 *Cylindrocarpon destructans*

1.4.1 *Cylindrocarpon* taxonomy

C. destructans has a global distribution, and is known to affect a variety of woody and herbaceous plants (Cabral *et al.*, 2012). Whilst a number of *Cylindrocarpon* spp. are considered minor pathogens, *C. destructans* is known to have a significant economic impact on crops such as ginseng (*Panax quinquefolium*), grapevines (*Vitis* sp.) and parsnips (*P. sativa*) (Samuels, 1990; Mantiri *et al.*, 2001).

Cylindrocarpon destructans (Zins.) Scholten is a soil-borne ascomycete from the Nectriaceae (Hypocreales) family, a group consisting of many species commonly associated with root rot disease symptoms on a range of hosts (Samuels & Brayford, 1990; Cabral *et al.*, 2012; Lombard *et al*., 2015). Many anamorphs, teleomorphs and synonyms exist for *Cylindrocarpon*, and *Cylindrocarpon*–like spp. This has led to some confusion in understanding and describing fungal species responsible for specific host symptoms (Lombard *et al*., 2015; Cabral *et al.*, 2012), such that now species identity is generally confirmed through molecular methods including PCR amplification of ITS (internal transcribed spacer) and β-tubulin regions.

Table 1.2 – Description of *C. destructans* synonyms, anamorphs and teleomorphs (adapted from Cabral *et al.*, 2012).

Species Name	Date	Synonym/Life Cycle Stage
Cylindrocarpon destructans (Zinssm.) Scholten var. destructans	2001	Anamorph
Neonectria radicicola (Gerlach & L. Nilsson) Mantiri & Samuels	2001	Teleomorph
Ilyonectria radicicola (Gerlach & L. Nilsson)	1963	Basionym (Teleomorph)
Cylindrocarpon radicicola Wollenw.	1924	Basionym
Ramularia destructans Zinssm.	1918	Basionym
Ramularia macrospora Wollenw.	1913	Basionym
Fusarium polymorphum Marchal.	1895	Basionym
Ramularia destructans Zinssm.	1918	Basionym

Due to the number of synonyms and anomorphs of *C. destructans* there have been many changes to the specific characteristics that define the species with the most recent and detailed study by Cabral *et al.* (2012). Conidiophores are mostly simple, being solitary or loosely aggregated, 1-4 septate, 40-80 µm long. Maroconidia formed on both simple and complex conidiophores are 1-3 septate, straight and cylindrical; microconidia are 0-1 septate, ellipsoid and straight. Conidia form in heads on simple conidiophores as white or unpigmented masses, with globose, brown, thick-walled, chlamydospores of 7-10 µm forming in chains or clumps (Cabral *et al.*, 2012).

1.4.2 *Cylindrocarpon* epidemiology

C. destructans is a soil-borne fungus present across diverse habitats and hosts, it is a rapidly growing fungus, capable of growing at low nutrient concentrations and at low oxygen levels; it has been found at a soil depth of up to 63 cm

(Garrido *et al.*, 2004; Matturi & Stenton, 1964). The species is thought to be a pioneer colonizer due to its rapid germination and mycelial growth, and its ability to use organic and inorganic nitrogen sources (Brayford, 1992). *Cylindrocarpon* survives in the soil as mycelium, conidia or chlamydospores, the latter of which can survive for several years (Taylor, 1964). When conditions are favourable, chlamydospores germinate, produce hyphae and grow towards host roots (Horsfall & Dimond, 1960). It is thought that the growing pathogen also produces conidia, which germinate in the soil increasing the inoculum potential (Horsfall & Dimond, 1960). It is thought that *C. destructans* enters roots through natural openings such as secondary root hairs, or through wounding from insects or nematodes (Scheck *et al.*, 1998). The disease is often observed affecting lines, or patches within a crop suggesting the infection spreads to neighbouring plants; this transmission could be through water distributing chlamydospores, or from mechanical or human activities (Maluta & Larignon, 1991).

1.4.3 *Cylindrocarpon* symptoms

C. destructans on parsnip causes blemishes and lesions to develop on the outer epidermis of the crown and shoulder of the root; according to the literature these can appear black/brown with orange flecks, similar to cankers caused by *Itersonilia* spp. or orange in colour (Channon & Thomson, 1981; McPherson, 2013). Whilst some cankers appear as bruises with undamaged epidermis tissue, orange/brown lesions appear 'corky', with subsurface tissue exposed as the infection has progressed deeper into the tissue.

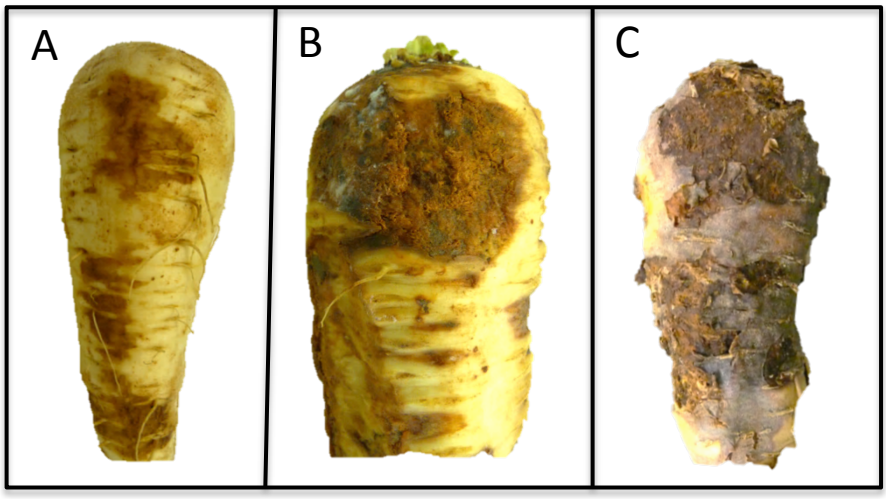


Figure 1.4 – *Cylindrocarpon destructans* symptoms. (A) *Cylindrocarpon destructans* infected parsnip root, orange/brown epidermal lesions. (B) *C. destructans* infected parsnip root, orange corky lesion. (C) *C. destructans* infected ginseng (*Panax quinquefolium)* root, orange/brown corky lesion

(Ministry of agriculture, food and rural affairs, 2012).

C. destructans symptoms on other host plants have similar symptoms of discoloured, disrupted tissues. In ginseng symptoms appear as orange/brown root rot that can affect both seedling and mature plants (Seifert *et al.*, 2003). In grapevine, spruce and pine species, black-foot disease is characterized by discoloured internal tissues, including the xylem (Rego *et al.*, 2001; Dumroese *et al.*, 2000). Unlike some pathogens, *Cylindrocarpon* appears to be isolated to the root system, and no foliar or above ground symptoms found in any host plant have been attributed to this pathogen (Dumroese *et al.*, 2000).

1.5 Other Parsnip pathogens

In addition to the three pathogens described above, there are a number of other fungal pathogens the literature cites as being a causative agent in parsnip canker. *Phoma* spp. were found to be a cause of canker by Channon (1963a), the casual agent of black canker, and similar in appearance to those attributed to

Itersonilia spp. In pathogenicity tests it was found to cause both root and foliar symptoms, although no foliar symptoms had been identified in the field (Channon, 1963a). Further studies by Cerkauskas (1985) and then McPherson (2013) also found *Phoma* spp., specifically *Phoma complanata*, to be causing lesions in parsnips.

Another key pathogen implicated in parsnip disease is *Pythium* spp. This oomycete has a wide host range affecting a number of economically important crops, causing a range of symptoms including root lesions and seedling damping-off (Minchinton, 2008). One study suggests *Pythium* spp. is most prevalent in parsnip crops during cold, wet periods when there is less competition from other fungal pathogens such as *Phoma*, *Fusarium* and *Alternaria* spp. (Petkowski *et al.*, 2010). Other studies suggest *Pythium* spp. act as a complex with *P. intermedium*, *P. ultimum*, *P. sulcatum*, *P. irregular* and *P. dissotocum* all being found within parsnip crops. However, *P. intermdium* and *P. sulcatum* were found to be the most pathogenic in glasshouse pathogenicity tests. It is also important to note that soil type, environmental conditions and crop management practices can all have an impact upon the *Pythium* spp. present (Petkowski *et al.*, 2013).

Other pathogens such as *Fusarium* spp., *Rhizoctonia* spp. and *Alternaria* spp. have been isolated from parsnip root disease; however, there is no current research describing the extent to which these pathogens are responsible for specific root symptoms and it is therefore likely they occur as secondary contaminants due to their increased frequency as soil temperature increases and rainfall decreases (Petkowski *et al.*, 2010). Given the range of species associated with root canker disease and the severity of the symptoms they cause, there is a need to implement cultural practices and control methods to reduce canker incidence and the associated crop losses.

1.6 Disease Control

In recent years the parsnip industry has reported high wastage figures due to parsnip canker with average crop losses of 20% (McPherson, 2013), although in some years this value has been significantly higher with growers reporting up to 80% crop losses (McPherson, 2013). These losses are generally associated with fungal pathogens including *I. pastinacae*, (Channon, 1963a) *M. acerina* (Channon, 1965) and *C. destructans* (Channon & Thomas, 1981). Changes in weather conditions are thought to have an impact on the prevalence of parsnip canker; it has been noted that cold damp seasons lead to a greater number of infections in both wild type and modern cultivars (Wilkinsons, 1952), and a high summer rainfall will directly correlate to an increase in canker incidence (Brown *et al.*, 1964). Channon (1964) suggested a number of cultural practices to aid in the prevention of parsnip canker. These included early sowings to encourage root establishment prior to winter, ensuring the crown/shoulder of the root is not exposed, and increasing spacing when sowing. These practices indicated a minor reduction in canker prevalence but did not result in an increase to crop yield (Channon, 1964; Brown & Nourish, 1964).

There are currently no specific fungicides licensed for the control of parsnip canker. There are, however, some fungicides available for use: Amistar (200 g / l azoxystrobin), Signum (26.7% w/w boscalid and 6.7% w/w pyraclostrobin) and Topsin (70% w/w thiophanate-methyl). These have shown promise in some early trials for canker control, but it was noted further work was required to detail the specific activity of the fungicide alongside other treatment options (Gladders *et al.* 1997). As an alternative to chemical control Gladders *et al.* (1997), tested 7 biopesticides including Prestop (500 g / 1000 L) and Serenade (200 ml / 1000 L) against *Cylindrocarpon*, *Pythium*, *Phoma* and *Fusarium* spp. None were shown to have a significant positive effect on reducing the development of canker symptoms.

As Itersonilia is a seed borne pathogen there have been some investigations to the potential of seed treatments in preventing the development of both foliar and root symptoms (Channon, 1969). However these have been inconclusive so far due to the phytotoxic effects of some fungicides (UK226: tebuconazole 2.75 g / 100,000 seed) combined with dry seasons leading to naturally low levels of Itersonilia (Gladders, 1997). Smith (1966) found stream-air treated seed at 45.5°C for 30 minutes was successful in removing Itersonilia from the seed coat without damaging the seed or reducing germination.

One of the current control methods advised for *M. acerina* is the implementation of crop rotation. However, as this pathogen has such a wide host range including crop and weed species, and the presence of long-lived survival structures in the form of chlamydospores, this method only slightly reduces the level of inoculum in the soil (Hermansen *et al.*, 1997). Research carried out into specific control methods for *C. destructans* on parsnip by McPherson (2013) found Amistar and Vivid (azoxystrobin & pyraclostrobin at 2, 20 and 100 ppm) showed good potential for controlling mycelial growth during agar-plate tests, and reduced damping-off in *in-vivo* pot trials, but suggested the need for further field trials due to low canker incidence (McPherson, 2013). As a result, there is a desire for alternative control such as plant resistance. Resistant lines to parsnip canker are commercially available, such as Avonresister, bred through continuous field selection for resistance to field canker caused by *Itersonilia, Mycocentrospora* and *Phoma* spp. However, market requirements for quality agronomic performance limit the cultivars available. As such, growers are dependant on the use of fungicides and crop management practices to reduce canker incidence (Gladders et al., 1997). However, modern breeding in large acre crops now utilises the latest technologies such as marker assisted breeding as described below. This project is extremely timely, as the new sequencing technologies allow us to move such practices into parsnip.

1.7 Plant breeding and marker assisted selection

Traditional plant breeding involves crossing an existing cultivar with a line possessing a desirable trait. This means whole genomes are crossed to produce progeny, which are then selected for according to the superiority of plants possessing the original trait of interest (Varshney *et al.*, 2007). It is therefore based on the phenotypic selection of superior genotypes within segregating progenies obtained from crosses (Francia *et al.*, 2005). Selection of superior plants involves visual assessment for agronomic traits, as well as laboratory tests for quality (Collard & Mackill, 2008). The size and composition of a plant population is an important factor in a breeding programme; the more segregating genes in a population the greater the population size in order to identify a specific gene combination. Typical breeding programmes may have hundreds of populations, and thousands of individual plants (Witcombe & Virk, 2001). For commercial seed companies, it will typically take up to 30 years to produce a new stable parsnip line (Sue Kenedy, 2012), so new technologies such as marker-assisted selection (MAS), are now being used in plant breeding to improve efficiency and precision (Collard & Mackill, 2008).

There are five main considerations for breeding companies on the use of MAS; reliability of markers linked to target loci, quantity and quality of DNA required, technical procedure for marker assays, high levels of polymorphism to discriminate between different genotypes and the cost-effectiveness of the technique (Mackill & Ni, 2000; Mohler & Sungrun, 2004). A range of markers can be used in MAS including simple sequence repeats (SSRs), sequence tagged site (STS), sequence characterized amplified region (SCAR) and single nucleotide polymorphisms (SNP) generated from specific DNA sequences of markers such as restriction fragment length polymorphisms (RFLPs) that are linked to a gene or quantitative trait locus (QTL).

Detection of genes or QTLs controlling traits is possible due to genetic linkage analysis based on the principle of genetic recombination during meiosis (Tanksley, 1993). Statistical methods such as interval mapping can be used to detect associations between DNA markers and phenotypic data. This identification of QTLs through DNA markers has been a major development in the characterization of quantitative traits (Paterson *et al.*, 1989).

Once markers that reliably predict trait phenotype have been identified they can be used for MAS, which speeds up the phenotype process by enabling seedling selection to be carried out. MAS also allows single plants to be selected, which can save time and resources in large-scale phenotype screening (Collard & Mackill, 2008). There are many applications of MAS in plant breeding. Among the most common in commercial settings are: marker-assisted evaluation of breeding material to assess cultivar purity, levels of genetic diversity for parental selection, study of heterosis for hybrid vigour and identification of shifts in allele frequencies within genomes (Xu & Crouch, 2008; Collard & Mackill, 2008), marker-assisted backcrossing to incorporate genes into an adapted or elite variety, marker-assisted pyramiding to combine several genes in one phenotype, early generation marker-assisted selection to eliminate plants with undesirable traits and focus attention on high-priority lines, and combined marker-assisted selection to maximise genetic gain through a combination of MAS and phenotype selection (Collard & Mackill, 2008).

MAS has greatly assisted in plant breeding. There are however disadvantages: the primary limiting factor to this system is the cost associated. However, new marker technology such as the application of next-generation sequencing (NGS) have provided high-throughput sequences for improved plant genotyping and breeding. This technology has led to the development of genotyping-by-sequencing (GBS) a more cost-effective technique that combines molecular marker discovery and genotyping (Mammadov *et al.*, 2012; He *et al.*, 2014). This project therefore aims to utilise this new marker technology to identify QTLs

conferring resistance to parsnip canker pathogens. This will result in improved screening for resistance in large parsnip breeding populations and speed up the process of commercialising new hybrid lines displaying resistance to canker pathogens.

1.8 Project Aims and Objectives

The overall aims of this project were to characterise pathogens responsible for causing parsnip canker and to develop tools to facilitate breeding for quantitative resistance to parsnip root canker diseases. The specific objectives were:

1. To determine the range of fungal pathogens causing root cankers on parsnips;

2. To characterise the diversity of *Itersonilia* spp., *M. acerina* and *C. destructans* isolates through molecular and biological approaches;

3. To determine the pathogenicity of *Itersonilia* spp., *M. acerina* and *C. destructans* isolates through the development of novel assays;

4. To develop screening tests to identify *I. pastinacae* and *M. acerina* resistance in parsnip breeding populations;

5. To analyse resistance within parsnip outbreeding mapping populations using QTL mapping and Next Generation Sequence analysis.

2. Causes of parsnip canker and other root symptoms

2.1 Introduction

Fungal pathogens have been identified as being the primary cause of parsnip (*Pastinaca sativa*) canker, which is a major contribution to the downgrading and rejection of parsnip crops, leading in some years to considerable economic losses (Gladders, 1997). The occurrence and type of symptoms can be highly variable depending on a number of factors, including cultivar, cropping history, weather conditions and previous disease incidence (McPherson, 2013). In the UK, fungal parsnip canker is responsible for average crop losses of 20%, often more severe in wet seasons, resulting from a greater number of spores being produced (Channon 1963b), whilst hot, dry conditions were found to retard the development of canker symptoms (Cerkauskas, 1985). Other countries have reported much greater losses, with Australia reporting up to 80% reduction in marketable yield in spring-harvested crops for the past 40 years (Minchinton, 2008).

There has been a general lack of research into the cause of parsnip canker, with Wilkinson (1952) first isolating *Itersonilia* spp. from parsnip canker. Further work by Channon (1956) described a range of pathogens as the causes of parsnip canker. More recently, the research of Gladders (1997) and McPherson (2013) gave an updated report on the causes of root blemishes in the UK which included *C. destructans*, *M. acerina*, *Fusarium* spp. and *Phoma* spp. However, these studies focussed on control strategies for the prevention and treatment of root canker pathogens. Channon (1963a) described two main types of canker typically present on the crown and shoulder of parsnip roots in the UK. These symptoms were: a black canker, caused by *Itersonilia pastinacae* (*I. pastinacae*), *Phoma* spp., or both; and an orange-brown canker of unknown cause. Results indicated pathogenic *I. pastinacae* was responsible for black cankers on 365 roots out of

581 roots sampled (63%), whilst pathogenic *Phoma* spp. was responsible for approximately 15% of symptoms. Further work by Channon (1963a) suggested a 'consortium' of fungi causing black cankers, while fungi isolated from brown cankers failed to induce similar symptoms. However, the development of brown cankers appeared to be associated with growth splitting, where root tissue was split open, generally as a result of irregular moisture conditions. Channon (1963a) used Koch's postulates to show that *I. pastinacae* isolated from brown cankers produced typical black canker symptoms following re-inoculation on parsnip.

Outside of the UK, *I. perplexans* has been implicated as the primary causal agent of parsnip canker in Australia (Minchinton, 2008), being responsible for 21.6% of positively identified isolations. In addition, a range of other fungal pathogens have also been identified as causal agents of parsnip canker in Australia. These include: *Fusarium* spp., *Cylindrocarpon* spp., *Mycocentrospora acerina* and *Phoma* spp*. Phoma* spp*.* were identified as being the primary cause of parsnip canker in Canada, with *Phoma complanata* identified as causing up to 80% of crop losses (Cerkauskas, 1985). This pathogen was reported to be a seed-borne pathogen and a strong correlation between severity of foliar symptoms and incidence of root canker was found (Cerkauskas, 1987). Despite having a narrower host range than *I. pastinacae*, *P. complanata* was found to be capable of over-wintering and surviving in soils for up to five months (Cerkauskas, 1987).

Mycocentrospora acerina has also been described as a causal agent of canker symptoms similar to those of *I. pastinacae* (Channon, 1965). Symptoms were generally described as black lesions, often surrounded by a brown/red ring. Channon (1965) found there was no difference in size or severity of symptoms on wounded or unwounded roots when inoculated with *M. acerina*, while the pathogen's wide host range (Hermansen, 1992) and capacity to grow and infect at temperatures below 0°C suggests an ability to develop symptoms under winter conditions (Channon, 1965). Outside the UK there is evidence of *M.*

acerina being isolated from Australian parsnip crops (Minchinton, 2008); however, despite its wide host range it has not been documented as a pathogen of parsnip in other countries.

Cylindrocarpon destructans has been identified as another fungus causing black/dark brown parsnip canker symptoms (Channon & Thomas, 1981). Isolates from lesions were confirmed as pathogenic and able to re-infect damaged/wounded parsnip roots, leading to the development of symptoms. This fungus has a narrower host range than *Itersonilia* spp. and *M. acerina*, and is generally considered to be a weak pathogen, particularly in relation to woody pine species, although it is considered to be a major pathogen of ginseng, grape vines and parsnip (Channon & Thomson, 1981; Lombard *et al.*, 2015; Cabral *et al.*, 2012). Whilst *C. destructans* has been identified as a parsnip pathogen in the UK and Australia, its global impact is more closely associated with infection of grape vines and ginseng (Probst, 2011).

More recently, *Pythium* spp. have also been implicated as part of a disease complex causing parsnip root rot. Parsnip and parsley (*Petroselinum crispum*, a related umbelliferous species) crops in south-east Australia suffer from 80-100% crop losses due to seedling damping-off, minor root lesions and mature root rot (Petkowski *et al.*, 2013). A total of nine *Pythium* spp. isolated from diseased roots were all found to be pathogenic upon inoculation of fresh parsnip tissue. Of the species isolated, several have been associated with a range of root symptoms in different plant species, including parsnip, in Canada, the Netherlands, South Africa, Germany and the UK (Petkowski *et al.*, 2013). In glasshouse pathogenicity tests, *Pythium sulcatum* and *Pythium intermedium* were found to be most pathogenic in causing damping-off symptoms in parsnip seedlings (Petkwski *et al.*, 2013).

Pathogen persistence in soils has been suggested as an important factor in the occurrence of parsnip root canker. The survival of *I. pastinacae* was first

demonstrated by Smith (1966), where the pathogen was found to survive in the soil in infected roots for at least 12 months. It was additionally found to survive as chlamydospores in the soil for at least 12 months in Australia (Minchinton, 2008). These results, alongside studies on *M. acerina* and *C. destructans* (Evenhuis, 1995; Minchinton, 2008), suggested that chlamydospores produced by *I. pastinacae*, *M. acerina* and *C. destructans* were able to survive in the soil for long periods, indicating that, without regular crop rotation with non-host plants, these pathogens can persist in causing infection (Minchinton, 2008).

In addition to fungal pathogens, *Chamaepsila rosa* (carrot root fly) is considered to be a major contributor to the incidence of parsnip canker within the UK. Larvae cause damage by tunnelling through the tissue, predisposing roots to fungal infections (Stone, 1954). Controlling carrot root fly incidence was also found to correlate with a significant reduction in parsnip canker incidence (Collingwood & Croxall, 1954). Carrot fly control usually involves pre-drilling soil prior to seeding, and treating crops with insecticides such as phorate and diazinon (Minchinton, 2008; Sivasubramanaim *et al.*, 1999). Carrot root fly has a global distribution, being found in North America, UK, Europe and Eurasia. Whilst not an issue in Australia, carrot root fly has also been documented in New Zealand, and it is therefore considered a major biosecurity issue for carrot and parsnip growers in Australia (Minchinton, 2008).

The most recent investigation into the range of root disease symptoms observed on parsnips in the UK was carried out by McPherson (2013) using samples from fields and packhouses in Norfolk and Yorkshire during the years 2010/2011. They identified 14 different types of symptoms from which seven different species of fungi were isolated, with *C. destructans* being frequently found (Table 2.1). The range of symptoms were significantly greater than previously identified on parsnips by Channon (1963a; 1963b; 1965) and, due to multiple organisms being recovered from lesions of each symptom type, it can be concluded that a

complex of fungal pathogens are responsible for parsnip canker and other root rot symptoms (Minchinton, 2008).

Table 2.1 – Root symptoms and associated isolated pathogens for field and packhouse parsnip roots in 2010/2011 (after McPherson, 2013).

Symptom	Organisms recovered
Crown canker	*Botrytis cinerea, Fusarium* sp.
Black crown lesion	*Cylindrocarpon destructans, Fusarium* sp., *Itersonilia* sp., *Verticillium* sp.
Black shoulder lesion	*Cylindrocarpon destructans*
Young red crown lesion (watery)	*Cylindrocarpon destructans, Fusarium* sp.
Young red shoulder lesion (watery)	*Botrytis cinerea, Fusarium* sp.
Red/brown mid-root canker	*Cylindrocarpon destructans*
Deep soft rot	*Cylindrocarpon destructans, Fusarium* sp.
Dry scars	*Botrytis cinerea, Cylindrocarpon destructans, Fusarium* sp., *Phoma* sp.
Dark narrow scars	*Cylindrocarpon destructans, Fusarium* sp.
Ginger blotch	*Botrytis cinerea, Cylindrocarpon destructans, Fusarium* sp., *Phoma* sp.
Red spots/speckles and stripes	*Cylindrocarpon destructans, Fusarium* sp., *Phoma* sp.
Cavity spot - like	*Botrytis cinerea, Fusarium* sp., *Cylindrocarpon* sp.
Red-root scars	*Cylindrocarpon destructans, Phoma* sp.
Black spots/scars	*Fusarium* sp.

Other than the McPherson (2013) study, little is understood about the extent and cause of parsnip canker within the UK. The aims of the work described in this chapter are:

- To collect samples of diseased parsnip roots;
- To isolate and identify the causal agents of parsnip canker using morphological and molecular techniques;

2.2 Materials and Methods

2.2.1 Sampling of *parsnip* roots

Diseased parsnip roots were obtained from locations throughout the UK as well as locations in Europe and one site in New Zealand (Table 2.2). The majority of samples were obtained by individual growers who were requested to collect diseased roots at separate points across a field. The numbers of roots received from a single location varied due to differences in disease incidence.

Table 2.2 – Country, location, sampling year and total number of diseased parsnip roots sampled in this study.

Country	Location	Sampling years	Roots sampled
England	Spalding, Lincolnshire	2012-13	47
England	Elsoms Seeds Ltd, Lincolnshire	2012-13	12
England	Redhill, Nottingham	2012-13	2
England	Spalding, Lincolnshire	2012-13	21
England	Newark, Nottinghamshire	2012-13	6
Spain	Valencia	2012-13-14	2
Sweden	Unknown	2013-14	2
Scotland	Cupar, Fife	2013-14	10
Netherlands	Unknown	2013-14	3
New Zealand	Ohakune, North Island	2014-15	2
		Total	107

Parsnips were washed and all symptoms subsequently photographed and described. Isolations of fungi were made by taking four 5 mm^2 plugs of tissue from the leading edge of the various lesion symptoms on each root and placing directly onto potato dextrose agar (PDA: Oxoid Ltd, UK), amended with chlorotetracycline hydrochloride (2 ml / L, Sigma-Aldrich, UK) to inhibit bacterial growth. Half of the tissue pieces were also surface sterilised with 70% ethanol v/v (EtOH) for three minutes to reduce contamination from bacteria, mites and nematode spp. To specifically isolate *Itersonilia* spp., four 5 mm^2 plugs of tissue were taken from the leading edge of the various lesion symptoms and stuck onto

the lid of Petri dishes with Vaseline (Sigma-Aldrich, UK). This is a standard approach to isolate slow-growing *Itersonilia* spp., as ballistospores are actively ejected onto the agar, hence avoiding competition from other fast growing fungi. In this case, tissue samples were not surface sterilised, to preserve ballistospores for colony development. Half of these tissue pieces were suspended over malt agar 2% (MA) while the other half were suspended over MA 2% with chlorotetracycline hydrochloride. All plates were incubated for 5-10 days at 20 °C and once mycelial growth was evident, fungal colonies were subcultured from the leading edge onto PDA, or MA if *I. pastinacae* had been provisionally identified, and incubated for 5-10 days at 20°C. Pathogens were initially identified through colony and spore morphology, and this was subsequently confirmed through PCR and sequencing.

2.2.2 Molecular identification of fungal isolates

Fungal isolates were subcultured from the leading edge of a culture onto PDA at 20 °C for 5-10 days to produce actively growing colonies. Three 5 mm^2 agar plugs were taken from the leading edge, placed into Petri dishes containing potato dextrose broth (PDB: Formedium, UK), and incubated at 20°C for 7 days. The agar plugs were then removed and the mycelial mat washed three times in sterile reverse osmosis (RO) water and blotted dry on tissue paper (KimTech: Kimberly-Clark Ltd, UK) before being freeze dried overnight. Genomic DNA was extracted from the freeze-dried mycelium using the DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. Isolates were initially identified by PCR amplification and sequencing of the internal transcribed spacer regions (*ITS*) of the ribosomal DNA (situated between the small-subunit and large-subunit rRNA genes). The 20 µl PCR reaction mixture consisted of 10 µl 1 x REDTaq Ready Mix PCR reaction mix (Sigma-Aldrich, UK), standard *ITS* primers: ITS1: TCCGTAGGTGAACCTGCGG, or ITS1F: CTTGGTCATTTAGAGGAAGTAA (a basidiomycete specific primer if *I. pastinacae* had been provisionally identified) with ITS4: TCCTCCGCTTATTGATATGC (White *et al*., 1990; Grades & Bruns, 1993)

(0.4 µmol L^{-1}) and approximately 10 ng DNA template. Thermal cycling parameters were 94 °C for 2 min; 40 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 60 s; 72°C for 10 min and then a hold at 12°C. Following PCR reactions, amplicons were visualised using gel electrophoresis with 1.5% agarose (Fisher Scientific) gels in tris-acetate-EDTA buffer, stained with 2 µl / 100 ml of GelRed (Biotium, UK). PCR purification was performed using the QIAquick PCR purification Kit (Qiagen), before 5 µl DNA template and 5 µl 5 pM primer template (ITS1 or ITS1-F as described above) were submitted for sequencing by GATC (Konstanz, Germany). BLAST analysis (Altshul *et al.*, 1990) was used to confirm species identity.

2.2.3 Classification of primary and secondary pathogens

Based on the number of organisms isolated from a single root symptom, they were described as either primary of secondary pathogens. Specifically, where a single organism was identified from multiple isolations of a single symptom, it was classified as a primary pathogen. However, where multiple organisms were identified from a single symptom, the most common according to the literature was defined as the primary pathogen, with the remaining organisms defined as secondary pathogens. However, where a single organism was identified from a single symptom on a root where carrot root fly (or other insect) damage was evident, and that organism hadn't previously been identified as a primary pathogen according to the literature, such as *Fusarium* spp., it was classified as a secondary pathogen.

In addition, to confirm pathogens isolated from roots were the causal agents of the symptoms observed, parsnip roots were re-inoculated with all primary pathogens, and a range of secondary pathogens.

2.3 Results

2.3.1 Identification of fungal isolates off diseased parsnip roots

A total of 107 diseased roots from England, Scotland, Spain, Sweden, The Netherlands and New Zealand were collected, from which 144 fungal isolates were obtained. *ITS* sequencing followed by BLAST analysis for each of these isolates identified a total of 22 fungal species (Table 2.3). The most frequent pathogens were *I. pastinacae*, *M. acerina*, *C. destructans* and *Fusarium* spp., identified from 8, 13, 44 and 50 isolates, respectively (Fig 2.1(A)). A range of other known plant pathogens were also identified, including: *Sclerotinia sclerotiorum*, *Botrytis* spp., *Pythium sulcatum* and *Phoma* spp., alongside general fungal saprophytes including *Scytalidium lignicola* and the microsporic fungus *Arthrobotrys*. As previously described, the pathogens were identified as primary or secondary pathogens based on the number of organisms derived from a single root symptom. *I. pastinacae*, *M. acerina* and *C. destructans* were identified as key primary pathogens, and to a much lesser extent a range of *Fusarium* spp. (Fig 2.1(B)). However, most Fusarium spp. were characterised as secondary pathogens along with some other fungal species, such as *S. sclerotiorum* and *P. sulcatum* as described above (Fig 2.1(C)). Furthermore, on a number of parsnip roots, insect damage was also evident (Table 2.3), including most roots from which *Fusarium* spp. and all other fungal species were identified as primary pathogens. The majority of roots exhibiting insect damage were sampled from the UK, although it should be noted root sampling was greater within the UK than other countries.

The majority of fungal species characterised as primary pathogens were identified from a range of locations and countries: *C. destructans* was identified in roots from multiple sites across England, as well as Scotland and New Zealand. *I. pastinacae* was identified in roots from England, Sweden and New Zealand, whilst *M. acerina* was identified across some sites in England only. A range of *Fusarium* spp. (Fig 2.2) were identified across all sampling sites, with the

exception of Sweden, whilst the remaining fungal species were predominantly identified from UK sites.

Table 2.3 – Root number, location, fungal species isolated and presence of insect damage for diseased parsnip roots from England, Scotland, Spain, Sweden, The Netherlands and New Zealand.

Root Number	Country	Location	Primary Pathogen	Secondary Pathogen	Insect Damage
1	England	Spalding, Lincolnshire	*Cylindrocarpon* sp.		
2	England	Spalding, Lincolnshire	*Cylindrocarpon* sp.		Y
2	England	Spalding, Lincolnshire	*Cylindrocarpon* sp.		
3	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		Y
3	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
4	England	Spalding, Lincolnshire	*Fusarium avanaceum*		Y
5	England	Spalding, Lincolnshire	*Fusarium latertium*		Y
5	England	Spalding, Lincolnshire	*Fusarium latertium*		Y
6	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
7	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
7	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*	*Fusarium solani*	Y
8	England	Spalding, Lincolnshire	*Phoma exigua*	*Fusarium oxysporum*	Y
9	England	Spalding, Lincolnshire	*Phoma exigua*	*Fusarium oxysporum*	Y
10	England	Spalding, Lincolnshire	*Phoma exigua*	*Fusarium oxysporum*	Y
11	England	Spalding, Lincolnshire	*Phoma exigua*	*Fusarium oxysporum*	Y
12	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		Y
12	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*	*Cylindrocarpon* sp.	
13	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*	*Cylindrocarpon* sp.	
14	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*	*Fusarium solani*	Y
14	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*	*Fusarium solani*	Y
15	England	Spalding, Lincolnshire	*Fusarium solani*		Y
15	England	Spalding, Lincolnshire	*Fusarium solani*		Y
16	England	Spalding, Lincolnshire	*Ilyonectria* sp.		Y
17	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		Y

Root Number	Country	Location	Primary Pathogen	Secondary Pathogen	Insect Damage
17	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		Y
18	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
19	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
20	England	Spalding, Lincolnshire	*Fusarium solani*		Y
20	England	Spalding, Lincolnshire	*Fusarium solani*	*Fusarium oxysporum*	Y
21	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
22	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
23	England	Spalding, Lincolnshire	*Botrytis* sp.		Y
24	England	Spalding, Lincolnshire	*Fusarium oxysporum*		Y
25	England	Spalding, Lincolnshire	*Sclerotinia sclerotiorum*		Y
25	England	Spalding, Lincolnshire	*Fusarium oxysporum*		Y
26	England	Spalding, Lincolnshire	*Phoma eupyrena*		Y
27	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*	*Phoma eupyrena*	
27	England	Spalding, Lincolnshire	*Scytalidium lignicola*		Y
28	England	Spalding, Lincolnshire	*Mycocentrospora acerina*		
29	England	Spalding, Lincolnshire	*Fusarium oxysporum*	*Fusarium verticilloides*	Y
29	England	Spalding, Lincolnshire	*Fusarium sacchari*	*Fusarium verticilloides*	Y
30	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
31	England	Spalding, Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	Y
31	England	Spalding, Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	Y
32	England	Spalding, Lincolnshire	*Fusarium solani*	*Fusarium lateritium*	Y
32	England	Spalding, Lincolnshire	*Fusarium solani*	*Fusarium oxysporum*	Y
32	England	Spalding, Lincolnshire	*Fusarium solani*	*Fusarium oxysporum*	Y
33	England	Spalding, Lincolnshire	*Fusarium solani*	*Fusarium oxysporum*	Y
34	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
34	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
35	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
36	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
36	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		

Root Number	Country	Location	Primary Pathogen	Secondary Pathogen	Insect Damage
37	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		Y
37	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		
38	England	Spalding, Lincolnshire	*Ilyonectria radicicola*	*Cylindrocarpon* sp.	Y
38	England	Spalding, Lincolnshire	*Ilyonectria radicicola*	*Cylindrocarpon* sp.	
39	England	Spalding, Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	Y
39	England	Spalding, Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	Y
40	England	Spalding, Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	Y
40	England	Spalding, Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	Y
41	England	Spalding, Lincolnshire	*Mycocentrospora acerina*		
41	England	Spalding, Lincolnshire	*Fusarium oxysporum*		Y
42	England	Spalding, Lincolnshire	*Fusarium solani*		Y
43	England	Lincolnshire	*Mycocentrospora acerina*		
44	England	Lincolnshire	*Cylindrocarpon destructans*		Y
45	England	Lincolnshire	*Phoma exigua*		
46	England	Lincolnshire	*Mycocentrospora acerina*		
47	England	Lincolnshire	*Mycocentrospora acerina*		
48	England	Lincolnshire	*Cylindrocarpon destructans*		
49	England	Lincolnshire	*Fusarium graminearum*	*Fusarium cerealis*	Y
50	England	Lincolnshire	*Mycocentrospora acerina*		
51	England	Lincolnshire	*Mycocentrospora acerina*		
52	England	Lincolnshire	*Fusarium avanaceum*		Y
53	England	Lincolnshire	*Mycocentrospora acerina*		
54	England	Lincolnshire	*Mycocentrospora acerina*		
55	England	Lincolnshire	*Fusarium avanaceum*	*Fusarium lateritium*	
56	England	Lincolnshire	*Cylindrocarpon* sp.		
57	England	Lincolnshire	*Fusarium verticilliodes*		Y
58	England	Lincolnshire	*Pythium sulcatum*		
59	England	Lincolnshire	*Epicoccum nigrum*		
60	England	Lincolnshire	*Mycocentrospora acerina*		
61	England	Lincolnshire	*Fusarium oxysporum*		Y

Root Number	Country	Location	Primary Pathogen	Secondary Pathogen	Insect Damage
62	England	Newark, Nottinghamshire	*Pythium sulcatum*	*Fusarium oxysporum*	
63	England	Newark, Nottinghamshire	*Pythium sulcatum*		
64	England	Newark, Nottinghamshire	*Fusarium oxysporum*		
65	England	Spalding, Lincolnshire	*Mycocentrospora acerina*		
66	England	Spalding, Lincolnshire	*Mycocentrospora acerina*		
67	England	Spalding, Lincolnshire	*Mycocentrospora acerina*		
68	England	Spalding, Lincolnshire	*Arthrobotrys conoides*		Y
69	England	Spalding, Lincolnshire	*Arthrobotrys conoides*	*Fusarium oxysporum*	Y
70	England	Spalding, Lincolnshire	*Fusarium solani*		Y
71	England	Spalding, Lincolnshire	*Cylindrocarpon* sp.		
72	England	Spalding, Lincolnshire	*Fusarium oxysporum*		Y
73	England	Spalding, Lincolnshire	*Fusarium solani*		Y
74	England	Spalding, Lincolnshire	*Cylindrocarpon destructans*		Y
75	England	Spalding, Lincolnshire	*Cylindrocarpon* sp.		Y
76	England	Spalding, Lincolnshire	*Fusarium solani*	*Fusarium oxysporum*	Y
77	England	Spalding, Lincolnshire	*Fusarium redolens*		Y
78	England	Spalding, Lincolnshire	*Fusarium redolens*		Y
79	England	Spalding, Lincolnshire	*Fusarium chlamydosporum*		Y
80	England	Spalding, Lincolnshire	*Mycocentrospora acerina*		
81	England	Spalding, Lincolnshire	*Itersonilia* sp.		
82	England	Spalding, Lincolnshire	*Itersonilia* sp.		
83	England	Spalding, Lincolnshire	*Botryotinia fuckeliana*		Y
84	England	Redhill, Nottingham	*Fusarium tricinctum*	*Fusarium avanaceum*	Y
85	England	Redhill, Nottingham	*Fusarium tricinctum*	*Fusarium avanaceum*	Y
86	England	Redhill, Nottingham	*Itersonilia* sp.		
87	Spain	Valencia	*Itersonilia* sp.		
88	Spain	Valencia	*Itersonilia* sp.		
89	Sweden	Sweden	*Fusarium tricinctum*	*Fusarium oxysporum*	Y
90	Sweden	Sweden	*Fusarium oxysporum*		Y
91	Scotland	Cupar, Fife	*Itersonilia* sp.		

Root Number	Country	Location	Primary Pathogen	Secondary Pathogen	Insect Damage
92	Scotland	Cupar, Fife	*Phoma* sp.	*Scytalidium lignicola*	Y
93	Scotland	Cupar, Fife	*Cylindrocarpon destructans*		
94	Scotland	Cupar, Fife	*Cylindrocarpon* sp.		
95	Scotland	Cupar, Fife	*Cylindrocarpon destructans*		
96	Scotland	Cupar, Fife	*Phoma* sp.	*Scytalidium lignicola*	
97	Scotland	Cupar, Fife	*Cylindrocarpon destructans*		
98	Scotland	Cupar, Fife	*Cylindrocarpon destructans*		
99	Scotland	Cupar, Fife	*Cylindrocarpon destructans*		
100	Scotland	Cupar, Fife	*Cylindrocarpon destructans*		
101	Scotland	Cupar, Fife	*Phoma* sp.	*Scytalidium lignicola*	Y
102	New Zealand		*Itersonilia*	*Fusarium oxysporum*	
102	New Zealand		*Itersonilia*		
102	New Zealand		*Itersonilia*		
102	New Zealand		*Itersonilia*	*Fusarium oxysporum*	
103	New Zealand		*Cylindrocarpon destructans*		
103	New Zealand		*Cylindrocarpon destructans*	*Fusarium oxysporum*	
103	New Zealand		*Cylindrocarpon destructans*		
103	New Zealand		*Cylindrocarpon destructans*		
104	Netherlands		*Cylindrocarpon destructans*		
105	Netherlands		*Cylindrocarpon destructans*	*Fusarium oxysporum*	
106	Netherlands		*Cylindrocarpon destructans*	*Fusarium chlamydosporum*	

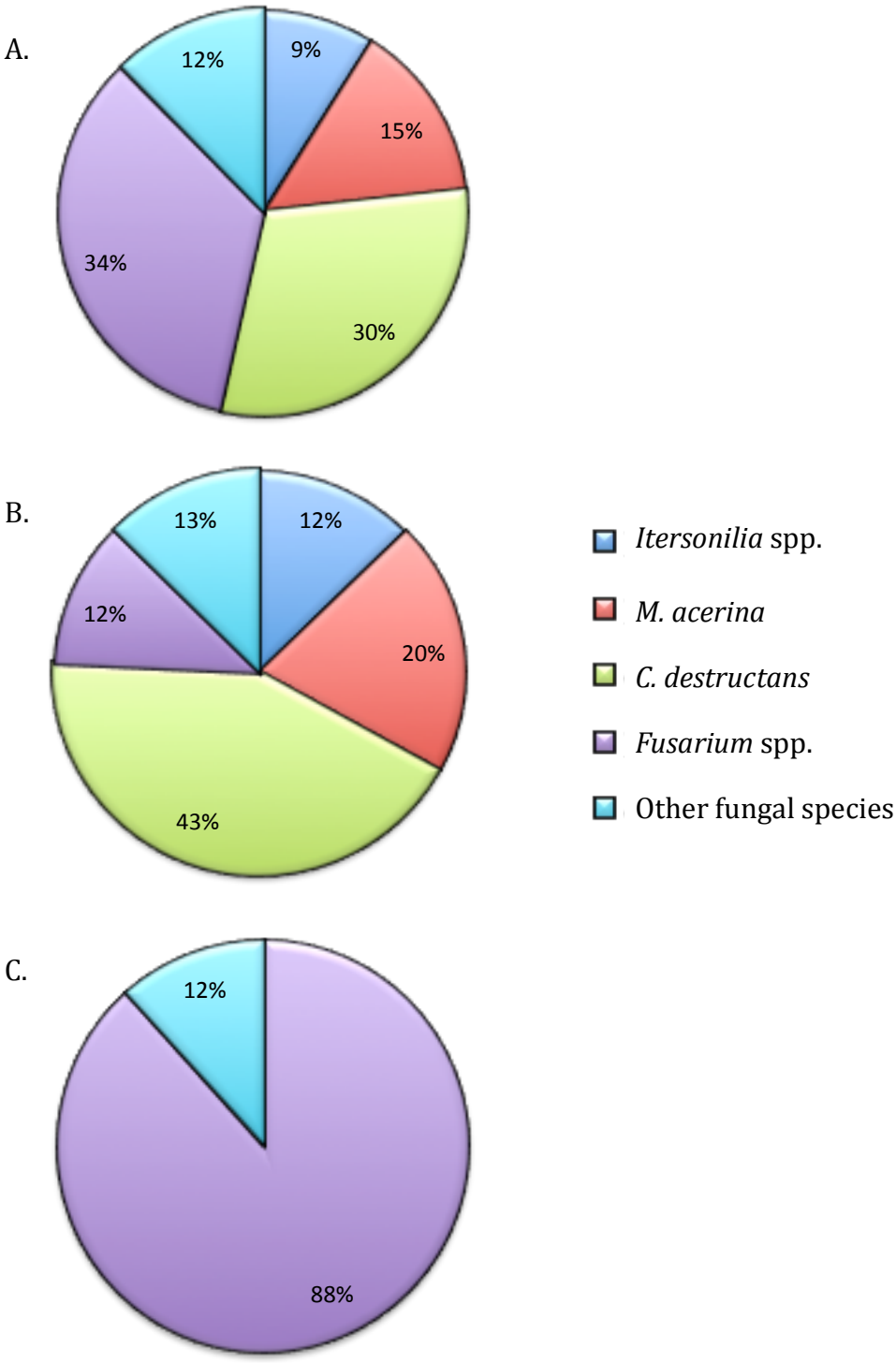


Figure 2.1 – Pie charts indicating percentage (%) of different fungal species isolated from diseased parsnip roots over all sampling sites. (A) Total percentage (%) of fungal species. (B) Percentage (%) of primary pathogens. (C) Percentage (%) of secondary pathogens.

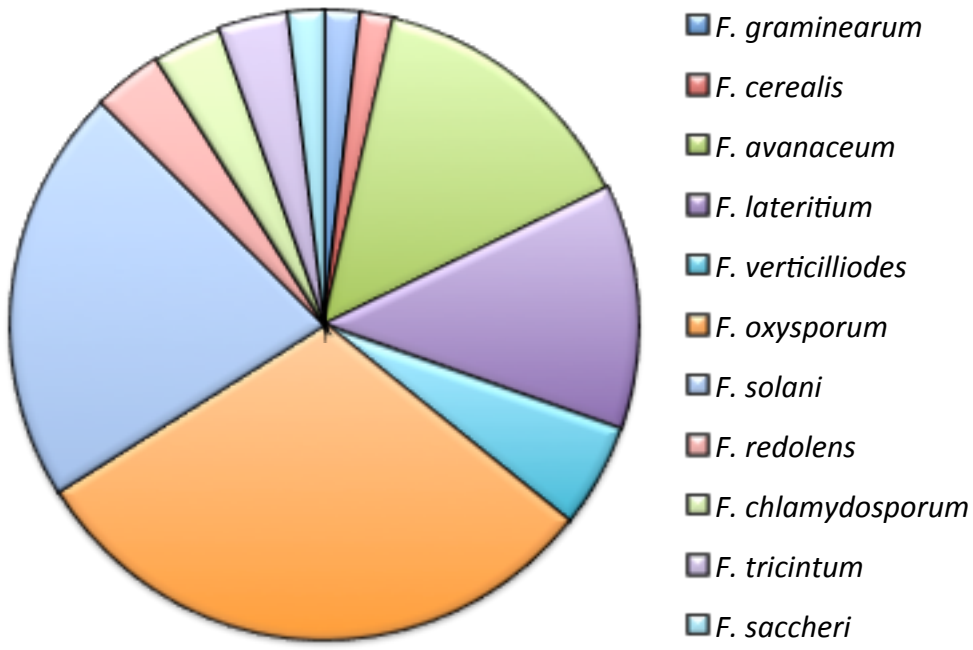


Figure 2.2 – Pie chart indicating the proportion of different *Fusarium* spp. isolated from diseased parsnip roots.

2.3.2 Description of symptoms from parsnip roots

From the 107 roots assessed for parsnip canker, six different symptom types were identified and associated with the respective causative pathogen (Table 2.4; Fig. 2.3). The roots displayed a range of black cankers of varying size and necrosis, which were caused by *I. pastinacae* and *M. acerina*. However, *I. pastinacae* infected roots generally had smaller black lesions with limited necrosis, whilst *M. acerina* developed larger black cankers with severe necrosis spreading to the core of the infected root and occasionally surrounded by a brown/black band. Small orange, and large orange corky lesions with varying degree of necrosis were both attributed to *C. destructans*, whilst superficial, 'cavity spot' type symptoms were caused by *P. sulcatum*. Although many *Fusarium* spp. were isolated, they frequently occurred as secondary pathogens and therefore were not clearly associated with any defined symptom type.

Where *Fusarium* was present, roots displayed high levels of necrosis and discolouration penetrating to the core of the root. Where other pathogens such as *Botrytis* and *Sclerotinia* spp. were the causal agent, the symptom was described as non-specific necrosis of brown/black/orange colouration, and relied on other indicators such as the presence of sclerotia to identify the causal agent from the symptom.

Table 2.4 – Description of symptoms and associated fungal pathogens isolated from diseased parsnip roots.

Symptom	Description	Primary pathogen	Secondary pathogen
1	Small hard black lesion	*I. pastinacae*	
2	Large soft black lesion	*I. pastinacae*	*Fusarium* spp.
3	Large soft black lesion	*M. acerina*	*Fusarium* spp.
4	'Cavity spot' like symptom	*P. sulcatum*	
5	Small orange lesion	*C. destructans*	*Fusarium* spp.
6	Large, corky orange lesion	*C. destructans*	*Fusarium* spp.
7	Black/orange, soft crown rot with sclerotia	*S. sclerotiorum* *Botrytis* spp.	*Fusarium* spp.
8	Black/brown/orange 'stripes'	*P. sulcatum*	*Fusarium* spp.
9	Large soft black/ brown/orange lesion	*Fusarium* spp.	'Other' spp.

Parsnip roots with symptoms not attributed to the major pathogens described above developed a range of canker-type lesions, which were brown/black/orange in colour and had varying degrees of necrosis, although most penetrated deep into the tissue to the core of the root. Often present was evidence of carrot root fly damage, where larvae feeding tunnels, and occasionally the larvae themselves, could be seen in the tissue. This damage was generally characterised by orange discolouration turning black with higher levels of root infestation.

There are a number of similarities between the results presented here (Table 2.4) and those observed by McPherson (2013) (Table 2.1), including black lesions caused by *I. pastinacae* and orange lesions caused by *C. destructans.* However, the majority of symptoms described by McPherson (2013) were attributed to more than one pathogen, with *Fusarium sp.*, being identified as responsible for all but one symptom type. By differentiating between primary and secondary pathogens, and identifying any other root damage such as that caused by carrot root fly, this work presents a more robust description of root symptoms and associated pathogens. This not only provides a more accurate understanding of the causes of root canker, but will also enable a more targeted approach for resistance breeding programmes and inform chemical applications and pest management approaches in the field.

Results from the re-inoculation of parsnip roots also confirms the descriptions of both symptoms and identification of primary and secondary pathogens (Table 2.4). All primary pathogen isolates, *I. pastinacae*, *C. destructans* and *M. acerina* were used to re-inoculate roots in addition to a range of secondary pathogens including *Fusarium spp*., *P. sulcatum* and *S. sclerotiorum*. Full details of re-inoculation with primary pathogens are described in later chapters; *I. pastinacae* (Section 3.2.2), *C. destructans* (Section 4.2.1) and *M. acerina* (Section 4.2.6).

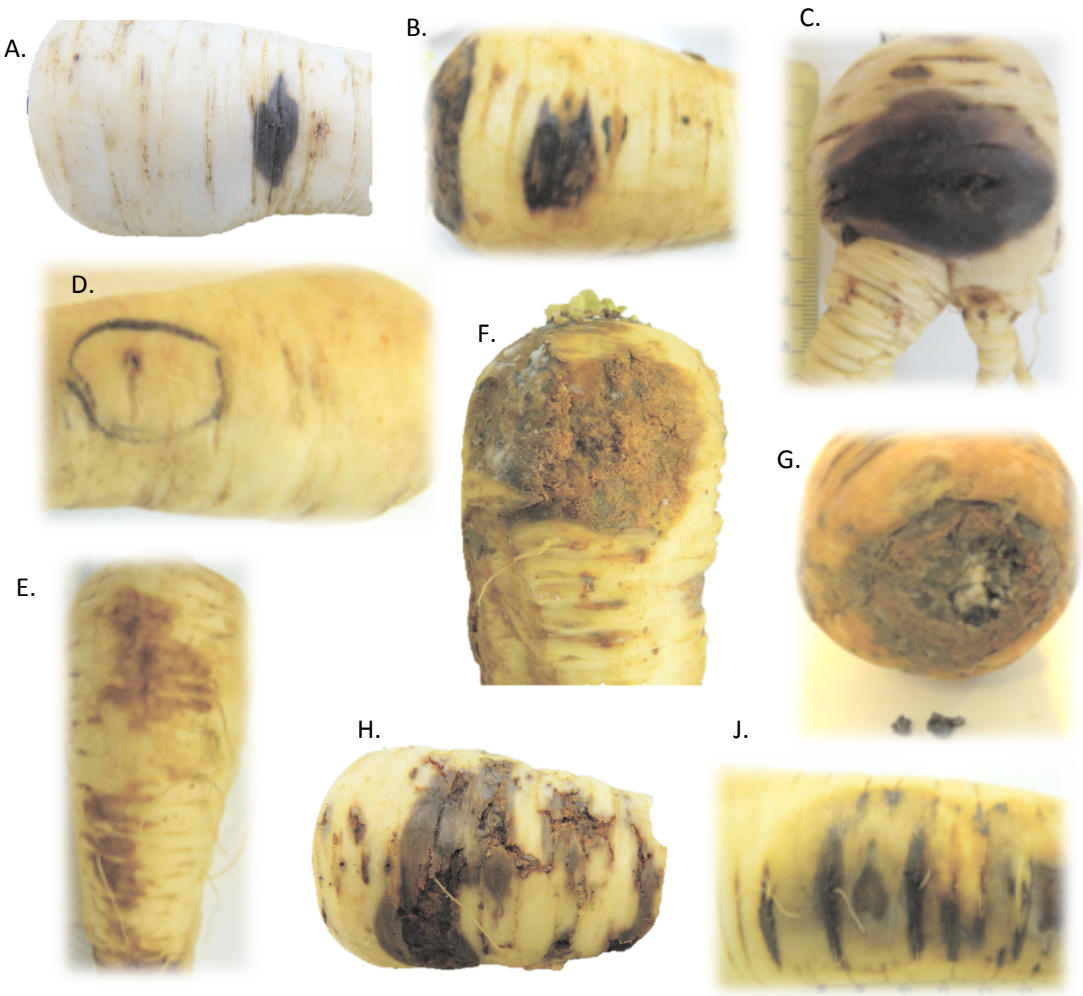


Figure 2.3 – Parsnip roots showing canker disease symptoms: (A) small black lesion caused by *I. pastinacae*; (B) Large black necrotic lesion caused by *I. pastinacae*; (C) Large brown/black lesion caused by *M. acerina*, surrounded by black/brown band. (D) Circled 'cavity spot' like symptom caused by *P. sulcatum*; (E) Small orange lesion due to *C. destructans*; (F) Large necrotic lesion caused by *C. destructans*; (G) Crown rot with white mycelium and sclerotia caused by *S. sclerotiorum*; (H) Orange/brown/black necrotic lesion with carrot root fly damage with *Fusarium* spp.; (I) Black/brown 'stripes' caused by *P. sulcatum* and *Fusarium* spp.

2.4 Discussion

Since the work of Channon (1963a; 1965; 1981) on UK parsnips, there have only been two recent studies examining the cause and incidence of parsnip canker, one in Australia (Minchinton, 2008) and another one in the UK (Table 2.1) (McPherson; 2013). The work undertaken here presents updated information on the types of root blemishes and symptoms currently observed by growers in the UK (Table 2.4) and for the first time the causal agents have been identified accurately through molecular methods, allowing a direct comparison between the current state of parsnip canker in the UK and that described previously.

I. pastinacae was first described as being the primary cause of parsnip canker by Channon (1963a), who identfied it as the causal agent of black canker symptoms on 63% of parsnip roots sampled. In contrast, the current analysis has found this pathogen to be responsible for only 15% of canker lesions. This is more in line with the recent work by Minchinton (2008) in Australia, where *I. perplexans* comprised 21.6% of pathogens identified from black cankers. In the present study, *I. pastinacae* was not isolated from roots in conjunction with any other pathogen and there was also no evidence of insect damage on roots from which *Itersonilia* spp. was isolated. Therefore, *I. pastinacae* was characterised as a primary pathogen and, although the number of infected roots was less than that reported by Channon (1963a), it remains a key pathogen of parsnip crops.

Channon (1963a) originally described root lesions caused by *I. pastinacae* as large dark brown, black or purplish-black rots, with penetration into the root usually less than 5 mm. He also noted that lesions normally occurred on the shoulders and crown of the root, but only occasionally on the shank. The colour and position of these canker symptoms are therefore comparable to those observed here, where *I. pastinacae* was isolated from black lesions found on the crown and shoulders of roots. This is also in line with the descriptions of Minchinton (2008) and McPherson (2013). However, in contrast to Channon

(1963a), *I. pastinacae* was mainly associated with small black cankers in the present study. These smaller lesions could be due to a combination of two factors; firstly, a potentially higher level of resistance in the varieties currently grown and bred since the work by Channon (1963a) which would potentially limit lesion expansion; secondly, an increased use of different control methods, including seed treatments to reduce seed-borne inoculum, general fungicide use to eliminate foliar symptoms, and crop rotation management, the combination of which might limit development of cankers by *I. pastinacae*.

I. pastinacae has been identified as a causal agent of parsnip canker in many other countries, including New Zealand, Australia, North America and Canada (Minchinton, 2008; McGovern *et al.*, 2006). Although the current study focussed mainly on sampling sites within the UK, the pathogen was also identified in samples from Spain, Sweden and New Zealand. This suggests that *I. pastinacae* is widely distributed as a primary causal agent of parsnip canker, although further work would be required to determine its abundance compared to other species causing root blemishes in other countries. However, as *I. pastinacae* is a seed borne pathogen, distribution of infected seed could explain the global spread of this disease.

M. acerina was also identified as a primary pathogen causing black canker on parsnip roots. It was originally described by Channon (1965) as responsible for large black/purplish-black lesions with exposed subsurface tissue as the infection progressed. In the current study, *M. acerina* was responsible for about 20% of total parsnip canker lesions but, unlike the work of Channon (1965), it was only isolated from small lesions perhaps due to the same reasons described above for *I. pastinacae*.

Moreover, *M. acerina* was only isolated from UK parsnip roots, although it has been documented as a causal agent of parsnip root rot in Australia, where it comprised 4.5% of total canker pathogens identified (Minchinton, 2008). The

global distribution of *M. acerina* as a parsnip pathogen has yet to be fully determined but, given the wide host range of the species, it is likely to be more prevalent than previously reported (Neergaard & Newhall, 1951).

Channon (1981) identified *C. destructans* as being the causal agent of black/brown lesions on UK parsnips, with orange flecks on the shoulders of the roots and also noted considerable associated damage by larvae of carrot root fly. Research by McPherson (2013) observed a more diverse set of symptoms associated with *C. destructans* on parsnip including black, brown, ginger and speckled lesions. Here, however, symptoms were more defined compared to these two previous studies, as *C. destructans* was only associated with small and large corky orange lesions. However, Minchinton (2008) identified *C. destructans* as being a major cause of parsnip canker, as it comprised 10.8% of total canker isolates in Australia, and the present analysis reveals that the situation is significantly worse in the UK, where *C. destructans* was identified as the causal agent for 40% of cankered roots. Channon (1981) also observed that *C. destructans* was isolated frequently from roots that had been damaged by carrot root fly larvae. Although this was also observed in the present study, *C. destructans* was considered to be a primary pathogen rather than an opportunistic saprophyte as it was associated with a large proportion of the lesions from undamaged roots. As for *M. acerina*, *C. destructans* also has a global distribution as a pathogen of other species, including grape vine and ginseng but its importance and distribution as a parsnip pathogen has yet to be determined.

Fusarium spp. comprised 18% of isolates identified from parsnip cankers in Australia (Minchinton, 2008), whilst in the UK, McPherson (2013) suggested that some *Fusarium* spp. were highly pathogenic on parsnip, and capable of causing lesions on undamaged roots. In this study, *Fusarium* spp. was identified as the causal agent for 11 of the 14 symptom types described by McPherson (2013; see Table 2.1), including a variety of black, red, brown and ginger symptoms. In the current study, *Fusarium* spp. were identified as associated with 7 of the 9 symptoms described (Table 2.4), including large and small black and orange

lesions. However, in contrast to the conclusion of McPherson (2013), the results strongly suggest that *Fusarium* spp. were predominantly secondary pathogens associated with primary infections by another fungal species or insect damage, such as that from carrot root fly larvae. However, some of the *Fusarium* spp. identified are known to infect plants; *F. graminearum*, *F. cerealis* and *F. verticilliodes*, are known cereal pathogens (Goswami & Kistler, 2004; Amarasinghe *et al.*, 2015) while *F. avanaceum*, *F. solani* and *F. oxysporum* are considered generalist pathogens, known to infect a wide range of plant hosts including tomato, potato and pea (Romberg & Davis, 2007). Some *Fusarium* spp. such as *F. oxysporum* are also divided into *formae speciales* based on the virulence of certain strains to a particular host or hosts (Correll, 1991). Moreover, many *F. oxysporum* strains are also non-pathogenic while there is also evidence that pathogenic *Fusarium* spp. and *F. oxysporum formae speciales* strains can colonise tissue of non-host plants (Banihashemi & Dezeeuw, 1973; Edel *et al.*, 1997; Coleman, 2008; Leoni *et al.* 2013). Identifying *Fusarium* spp. as primary pathogens can therefore be complex but as no single species was found consistently to cause the same root symptoms, and most *Fusarium* isolates obtained were associated with root damage, it is highly likely that they are saprophytes, colonising parsnip tissue opportunistically. However, it is clear that *Fusarium* spp. have a secondary impact on the quality of parsnip roots.

Incidence of *Pythium* spp. on diseased parsnip roots was low, with only three isolates identified from two different sites in the UK. All three isolates were identified as *P. sulcatum*, which has been previously implicated in parsnip root rot in Australia (Petkowski *et al.*, 2013), alongside a range of other *Pythium* spp. The symptoms attributed to *P. sulcatum* in the parsnip root were characterised as 'cavity spot' like, being very similar to symptoms caused by the same pathogen in carrot roots. Cavity spot in carrot is generally attributed to *P. violae*, although a range of *Pythium* spp. have been implicated, including *P. sulcatum*, *P. intermedium* and *P. sylvaticum* (White, 1985). Previous work by Petkowski *et al.* (2013) identified up to 11 different *Pythium* species associated with parsnip

blemishes, including *P. sulcatum*, *P. intermedium* and *P. sylvatium*, whilst globally *Pythium* spp. have been associated with root rot complexes in umbelliferous herbs such as parsley (Petkowski *et al.*, 2013). Due to the low number of isolates identified in this study, *Pythium* spp. were not considered to be a primary pathogen of parsnip. However, as the majority of parsnip growers will also grow carrots, and there is a clear link between *Pythium* spp. causing cavity spot in carrots and blemishes in parsnip roots, there is a need to implement good crop rotation and control methods to ensure infection in one crop does not lead to infection of a different crop in the same site the following year.

Numerous other fungal species were also isolated from diseased parsnip roots sampled in the UK, including known plant pathogens such as *Botrytis* spp. and *S. sclerotiorum*. These two species have a global distribution and are known pathogens on a wide range of host plants (Elad *et al.*, 2007; Bolland & Hall, 1994). They were also isolated principally from large black/orange soft crown rots, often penetrating deep into the parsnip tissue. As both species produce airborne spores, it is likely that infection originally arose in the foliage with subsequent growth of the pathogens into the crown, as has been observed for *S. sclerotiorum* in carrot, while carrot fly damage may also have facilitated their entry.

The original work on the causes of parsnip canker by Channon (1963a) identified *Phoma* spp. as being another key causal agent of black/brown cankers on parsnip roots. The lesions were characterised as having brown centre and pycnidia either on the surface of, or embedded in, the lesion. In Australia, Minchinton (2008) also identified *Phoma* spp. as being responsible for 3.6% of isolates obtained from canker-like symptoms in parsnips, whilst McPherson (2013) frequently isolated *Phoma* spp. from a range of symptoms, including dry scars, ginger blotch and red speckles. However, in the current research, *Phoma* spp. was isolated only in UK samples and from just seven root lesions characterised by large black/brown orange blemishes penetrating deep into the tissue. The *Phoma* spp.

were also generally isolated from roots where *Fusarium* spp. and insect damage were also present. Overall, this indicates that the frequency of *Phoma* spp. associated with parsnip canker is significantly lower than previously reported by the other authors.

The work of Channon (1963a) was seminal in identifying *I. pastinacae* as the primary cause of canker in parsnip roots while further work also indicated the potential importance of *C. destructans, M. acerina* and *Phoma* spp. (Minchinton, 2008; McPherson, 2013). Since then, the only other study identified *C. destructans* as more widely associated with different parsnip root blemishes (McPherson, 2013). However, for the first time in this study, particular canker symptoms have been clearly associated with certain pathogens, which have also been identified by molecular methods. This has therefore provided a more robust basis for identifying the causal agents of the different types of parsnip canker. *C. destructans* and *M. acerina* and, to a lesser extent, *I. pastinacae* were the main species causing parsnip canker, and this provides important information for growers, breeders and the wider industry. Most growers still attribute all black cankers to *I. pastinacae* (personal communication, Elsoms Seeds Ltd, 2013), although it is clear now that it is not as predominant as the other two pathogens. Based on this information, control approaches and breeding programmes can now be better targeted in order to provide more effective control of parsnip canker.

3. Characterisation of *Itersonilia* spp. isolates from parsnip

3.1 Introduction

3.1.1 Itersonilia species taxonomy

As a basidiomycete, *Itersonilia* spp. are in the order Cystofilobasidiales, a relatively newly described group containing primarily yeasts from cold regions (Millanes *et al.*, 2011; Fell *et al.*, 1999). These psychrophiles have the ability to grow and reproduce at low temperatures of around 0°C, with some species such as *Mrakiella cryoconiti* and *Mrakia frigida* maintaining growth at -12°C (Margesin & Fell, 2008; Ming-xiu and Pei-jin, 2007). These yeasts have been found in numerous low temperature environments such as Antarctic snow or soil, subglacial sediments and ice. *Itersonilia* spp. and other Cystofilobasidiales have also been identified in frozen food products such as fish and meat (Margesin & Fell, 2008; Dorn-In *et al.*, 2013) while species such as *Cryptococcus* spp. have been linked to infection in mammals such as the Californian sea lion (*Zalophus californianus*) and humans (Field *et al.*, 2012; Byrnes *et al.*, 2011). The Cystofilobasidiales are therefore a broad order, containing a range of organisms from a variety of habitats, and infecting very diverse hosts.

3.1.2 Itersonilia species and dimorphism

As previously set out in Chapter 1, three *Itersonilia* spp. have been described: *I. perplexans*, *I. pastinacae* and *I. pyriformis*. However, research by Boekhout (1991b) concluded that *I. perplexans* and *I. pyriformis* represented one species. According to Channon (1956), *Itersonilia* spp. produce both ballistospores, a spore discharged into the air from a sterigma tip, and chlamydospores, thick-walled resting spores. All *Itersonilia* spp. are known to produce ballistospores, however it is the presence or absence of chlaymdospores that currently

differentiates between the three species. *I. pastinacae* differs only slightly from *I. perplexans* in that it produces abundant chlamydospores and is specific to parsnip. However, it was still observed that some parsnip isolates did not produce chlamydospores while isolates cultured from other hosts did (Channon, 1963b).

All *Itersonilia* spp. are dimorphic, growing in nature as dikaryotic hyphae, but in laboratory cultures can develop as monokaryotic hyphae with yeast cells appearing after an initial phase of mycelial growth (Boekhout *et al.*, 1991a). The monokaryotic yeast phase can arise under conditions of high relative humidity or when the culture is grown submersed (Sowell & Korf, 1960), but has never been observed in nature. It has been suggested therefore that this may indicate the yeast cells have a low fitness or a narrow ecological amplitude (Boekhout *et al.*, 1991a). The yeast phase of an *I. perplexans* isolate was demonstrated to be non-pathogenic on chrysanthemum, whilst plasmogamy, the fusion of two parent cells resulting in a single cell with two separate nuclei, has been observed between yeast isolates of *I. pastinacae* and both *I. pyriformis* and *I. perplexans* (Boekhout *et al.*, 1991a). Dimorphism refers to the ability of certain fungi to switch between unicellular yeast and multicellular filamentous yeast growth forms in relation to changing environmental cues (Nadal *et al.*, 2008). Changes in the environment generate a variety of detectable signals. Dimorphic fungi perceive these signals and may undergo the morphogenic shift to adapt to new conditions. The type and intensity of the signals resulting in the switch varies with fungal species. *Mucor* species exhibit dimorphism in response to the carbon source available, and the level of oxygen in the atmosphere (Orlowski & Ross, 1981). In other fungal species such as *Ustilago maydis*, the cause of corn smut, the switch is not reversible, with mating and completion of the life cycle required to regain a particular growth form (Nadal *et al.*, 2008).

Ustilago maydis is often considered a model organism for the study of fungal dimorphism and pathogenesis. It can exist as both budding cells and true hyphae

and exhibits a number of genetic properties making it an excellent candidate to study the molecular mechanisms of dimorphism (Bölker, 2001). During the life cycle of *U. maydis* non-pathogenic haploid cells, showing yeast-like growth have to fuse to form dikaryotic hyphae, which are then able to infect maize plants (Kahmann & Bölker, 1996). The dimorphic switch and transition between the non-pathogenic and pathogenic lifecycle are regulated by a single master control locus that encodes pair of transcription factors (Bölker, 2001). Haploid wild-type strains of the fungus grow as budding yeasts and are saprobic, upon mating a filamentous dikaryon is produced and the fungus converted to an obligate parasite. Only during the final stage of development does the fungus undergo morphological changes associated with colonisation of the host environments, and produce diploid spores (Garcia-Pedrajas et al., 2009). Key transcription factor within *U. maydis* have been identified as being associated with the dimorphic switch and virulence including the MAPK cascade and the cAMP signalling pathways, where it was found distruption of these signalling pathways severely reduced the virulence of isolates (Bölker, 2001).

Generally, dimorphism, sexual development and virulence are tightly associated in the life cycles of many dimorphic fungi (Madhani & Fink, 1998). Whilst no research has been carried out into the specific signals that trigger the switch between hyphal and yeast stages in Itersonilia, the cell signalling pathway network in other dimorphic pathogens is well conserved. Two main pathways have been identified: the cAMP-dependant protein kinase A (PKA) and the mitogen-activated protein kinase (MAPK) (DeVoti *et al.*, 1991; Mochizui & Yamamoto, 1992; Neiman *et al.*, 1993). These pathways are not exclusive to pathogenic dimorphic fungi, also appearing in non-pathogenic fungi and yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Pan *et al.*, 2000; Schwartz & Madhani, 2004).

3.1.3 *Itersonilia pastinacae* and epidemiology of *Itersonilia* spp.

As described in Chapter 1, *I. pastinacae* was first described by Channon (1956) as a seed-borne plant pathogen causing black canker on parsnip roots as well as black/brown necrotic lesions surrounded by a green-yellow halo on leaves, petioles and inflorescences. Despite having a wide host range and distribution, little research has been carried out on *I. pastinacae*, particularly in relation to pathogenicity on parsnip and molecular characteristics.

Research on *I. perplexans* on edible burdock (*Arctium Lappa L*.) found that an increased inoculum density resulted in an increased rate of disease development, with black streaks on leaves increasing in a linear fashion as the inoculum density increased from 1 x 10^2 to 1 x 10^6 ballistospores / ml. It was also found that burdock leaves less than five day old were more susceptible than older leaves approximately 30 days old, which were essentially immune to infection (Horita & McGovern, 2005). Research identified peak Itersonilia outbreaks in edible burdock to occur during periods of low temperature and high rainfall, whilst peak parsnip canker incidence correlated with high rainfall during June to September in the UK (Brown *et al.*, 1964; Horita & Yasuoka, 2002). In addition to umbelliferous crops, *Itersonilia* spp. are also a pathogen of the Asteracae family, including sunflower, chrysanthemum, anemone and dahlia, causing petal blight (Dosdall, 1956; Sackston, 1958). Work by McGovern *et al.* (2006) on petal blight of China aster confirmed growth, sporulation and infection processes of Itersonilia were favoured by high relative humidity (>70%) and cool temperatures (10-15 °C). Whilst at cut-flower production sites, post-harvest petal blight frequently occurs, with Gandy (1966) reporting symptoms developing between 1 °C, when cut flowers were held under refrigeration, and 21 °C. It was also noted that low temperatures failed to halt growth or sporulation, with infected inflorescences of chrysanthemum being found after 31 days of frosts. Itersonilia has also been reported to survive in the UK between crops on plant debris, as well as saprophytically on many weeds and cultivated

plants, including kale and broccoli, indicating the species is widely distributed (Gandy, 1966).

Work by Ingold (1984) found Itersonilia grown on malt agar at 20°C has a radial extension rate of about 2 mm day^{-1}, that continued until the Petri dish was covered. In contrast, the growth rate of the yeast phase was significantly reduced, occurring after 2-4 weeks of mycelial growth and at only 0.2 mm day^{-1} at 20°C, with the colony rarely extending beyond a diameter of 10 mm. Ingold also reported that, unusually, these yeast-type cells, particularly those grown in darkness, occasionally gave rise to ballistospores, although this was not consistent across all isolates.

No research has been carried out on the ability of different *Itersonilia* spp. to produce spores, although research into spores found chlamydospores to be long-lived survival structures, with evidence they can survive from one year to the next in the soil (Smith, 1966). However, ballistospore inoculation of burdock leaves at 10-15°C led to the highest levels of infection, compared to other higher and lower temperatures (Horita *et al.*, 2005). Channon (1963b) suggested that infected parsnip leaves are not only a phase in the spread of the pathogen but also a source of spores for root infection. Therefore, based on this information, it could be hypothesised that ballistospores primarily infect foliage during warmer temperatures, when new leaves and seed stock are growing. When the foliage starts dying, the fungus produces increased numbers of chlamydospores, for both infecting parsnip roots and as survival structures for winter.

There is currently very limited understanding of the diversity of *I. pastinacae* from parsnip and other hosts, and little can be inferred about the pathogen from the knowledge of other species in the Cystofilobasidiales order. Therefore, the main aim of this work was to study the biological and molecular characteristics of *I. pastinacae* isolates. The specific objectives were:

- To determine the pathogenicity of different *I. pastinacae* isolates from parsnip and *Itersonilia* spp. from other hosts on parsnip roots and leaves;
- To determine the effect of temperature on mycelial growth and spore production of different *I. pastinacae* isolates from parsnip and *Itersonilia* spp. from other hosts;
- To investigate intra-species diversity within different *Itersonilia* spp. using housekeeping and functional gene analysis;
- To investigate the up-regulated and down-regulated genes relating to the fungal and yeast phases of *Itersonilia* spp. using transcriptome sequencing and gene expression analysis;
- To develop a species specific PCR assay for the detection of *Itersonilia* spp.

3.2 Materials & Methods

3.2.1 Collection of *Itersonilia* isolates.

A total of 51 *Itersonilia* spp. isolates were collected from various locations and hosts (Table 3.1). In addition to those cultures isolated from diseased parsnip roots as described in Chapter 2, cultures were also isolated from infected parsnip seed and foliage of parsnip and other host species. *Itersonilia* spp. was isolated from this diseased material through suspending infected seed or 0.5 cm^2 sections of infected leaf material above malt agar (MA) as described in Chapter 2. Cultures were incubated at 20°C for five days before being subcultured for storage on 0.2% MA and Czapek Dox Agar (CDA) slopes at 4°C or as frozen mycelial MA (0.2%) blocks in Potato Dextrose Broth (PDB) amended with 20% glycerol at -20°C for longer term storage.

Table 3.1 – Isolate number, host and origin of *Itersonilia* spp. isolates characterised in this study.

Culture code	Fungal species	Host	Origin	Country
IP1	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP2	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP3	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP4	*I. pastinacae*	Parsnip Seed	NIAB/Elsoms	England
IP5	*I. pastinacae*	Parsnip Seed	NIAB/Elsoms	England
IP6	*I. pastinacae*	Parsnip Seed	NIAB/Elsoms	England
IP7	*I. pastinacae*	Parsnip Seed	Plant Health Services	England
IP8	*I. pastinacae*	Parsnip Seed	Plant Health Services	England
IP9	*I. pastinacae*	Parsnip Seed	Plant Health Services	England
IP10	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP11	*Itersonilia* sp.	Parsnip Root	Stockbridge House	England
IP12	*Itersonilia* sp.	Parsnip Root	Stockbridge House	England
IP13	*Itersonilia* sp.	Chrysanthemum	Stockbridge House	England
IP14	*Itersonilia* sp.	Chrysanthemum	Stockbridge House	England
IP15	*Itersonilia* sp.	Dill	Stockbridge House	England
IP16	*Itersonilia* sp.	Dill	Stockbridge House	England
IP17	*Itersonilia* sp.	Dill	Stockbridge House	England
IP18	*I. pastinacae*	Parsnip Seed	Elsoms	England

Culture code	Fungal species	Host	Origin	Country
IP19	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP20	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP21	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP22	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP23	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP24	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP25	*I. pastinacae*	Parsnip Seed	Elsoms	England
IP26	*Itersonilia* sp.	Chrysanthemum	ADAS	England
IP27	*I. pastinacae*	Parsnip Leaves	Elsoms	England
IP28	*I. pastinacae*	Parsnip Leaves	ADAS	England
IP29	*I. pastinacae*	Parsnip Root	VCS	England
IP30	*I. pastinacae*	Parsnip Root	Elsoms	England
IP31	*I. pastinacae*	Parsnip Seed	Elsoms	New Zealand
IP32	*I. pastinacae*	Parsnip Seed	Elsoms	New Zealand
IP33	*I. pastinacae*	Parsnip Seed	Elsoms	New Zealand
IP34	*I. pastinacae*	Parsnip Seed	Elsoms	New Zealand
IP35	*I. pastinacae*	Parsnip Leaves	Nottingham	England
IP36	*I. pastinacae*	Fennel	Middlesex	England
IP37	*I. pastinacae*	Dill	Middlesex	England
IP38	*I. pastinacae*	Parsley	Middlesex	England
IP39	*Itersonilia* sp.	Parsnip Root *	Cupar, Fife	Scotland
IP40	*Itersonilia* sp.	Parsnip Root *	Elsoms	Sweden
IP41	*Itersonilia* sp.	Parsnip Root *	Nottingham	England
IP42	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP43	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP44	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP45	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP46	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP47	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP48	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP49	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP50	*I. pastinacae*	Parsnip Seed	Elsoms	France
IP51	*I. pastinacae*	Parsnip Seed	Elsoms	France

* Indicates isolates collected from root sampling in Chapter 1 (Table 2.3)

3.2.2 Pathogenicity of *Itersonilia* spp. isolates on parsnip roots

Experiments were set up to assess the pathogenicity of all *Itersonilia* spp. isolates (excluding IP14, IP16 and IP18; see Table 3.1) on freshly harvested parsnip roots from a commercial grower. Parsnip roots (cv. Picador) were mechanically harvested, washed with tap water, surface sterilized with 70% ethanol (v/v) and air-dried. Each root was checked for any disease symptoms before the widest part of the root was inoculated with a 5 mm agar plug of each *Itersonilia* spp. isolate from a twelve-day-old actively growing colony. Parsnips were then incubated at 12°C on damp tissue in sealed 3 L plastic boxes and moist conditions maintained to encourage disease development by misting roots with sterile RO water once a week. Six replicate parsnip roots were set up for each *Itersonilia* spp. isolate, and three repeat experiments carried out. Photographs of symptoms were taken weekly from three weeks post inoculation and lesion area measured using ImageJ (https://imagej.nih.gov/ij/).

3.2.3 Pathogenicity of *Itersonilia* spp. isolates on detached parsnip leaves

Experiments were set up to assess the pathogenicity of *Itersonilia* spp. on detached parsnip leaves. The same set of *Itersonilia* spp. isolates were used as those in the root assays (Section 3.2.2, Table 3.1). Parsnip plants (cv. Panache), were grown from seed in a 2:1 mix of Levington F2 compost and sharp sand (Everris, UK) in 2 L pots and placed in a glasshouse compartment at 20°C. When the plants were sixteen weeks old, leaves were removed for inoculation. *Itersonilia* spp. isolates were subcultured by placing a 5 mm mycelial plug taken from the edge of an actively growing colony in the centre of a Petri dish containing 25 ml of 0.2% MA. Plates were sealed with parafilm and incubated at 20°C for 14 days, after which 1 ml sterile RO water was added and spores gently removed from the fungal colonies using a spreader. The resultant spore suspensions were filtered through a milk filter (190 mm) (Goat Nutrition Ltd, UK) to remove mycelium and spore numbers determined using a haemocytometer and adjusted to 1×10^5 spores mL^{-1}. Detached parsnip leaves were placed into 1 L

plastic boxes on damp tissue and inoculated with 2 x 20 µl drops of spore suspension, one each side of the central vein. Boxes were sealed and placed in controlled environment at 20°C with a 16 h photoperiod. Boxes were removed after seven days and photographs of the foliar symptoms were taken. Lesion area was then measured using ImageJ (https://imagej.nih.gov/ij/). For each *Itersonilia* spp. isolate twelve detached leaves were inoculated. Four repeat experiments were carried out.

3.2.4 Effect of temperature on *Itersonilia* spp. growth rate

Experiments were set up to determine the effect of temperature on the growth rate of *Itersonilia* spp. isolates on MA using the same set of isolates selected for the pathogenicity experiments (Section 3.2.2, Table 3.1). Petri dishes containing 25 ml of MA were centrally inoculated with a 5 mm mycelial plug taken from the actively growing edge of each *Itersonilia* spp. isolate. Plates were sealed with parafilm and placed into incubators at 0, 5, 10, 15, 20 and 25°C. A total of four replicate plates per isolate were prepared and growth measurements of colony diameter at two perpendicular points were taken twice weekly for a period of thirty days. Following these experiments, *I. pastinacae* isolate IP10 was selected as a standard isolate to test the effect of additional temperatures of 2.5, 17.5, 22.5, 27.5 and 30°C in order to perform more accurate parametric model fitting. As above, there were four replicate plates per isolate with twice weekly measurements of growth, for a total of 30 days.

3.2.5 Effect of temperature on *Itersonilia* spp. spore production

Experiments were set up to determine chlamydospore and ballistospore production of *Itersonilia* spp. isolates at different temperatures using the same set of isolates as described in the pathogenicity experiments (Section 3.2.2, Table 3.1). A 5 mm mycelial plug taken from the edge of an actively growing plate was placed in the centre of Petri dishes containing 25 ml MA. Plates were placed into incubators at 0, 5, 10, 15, 20 and 25°C in darkness for fourteen days, after which

1 ml sterile RO water was added and spores gently removed from the fungal colonies using a sterile spreader. The spore suspension was filtered through a milk filter (Goat Nutrition Ltd, UK) to remove mycelium and the number of chlamydospores and ballistospores determined using a haemocytometer. Four replicate plates were set up per isolate at each temperature, with ten haemocytometer counts per Petri dish.

3.2.6 Genome sequencing and assembly of *I. pastinacae*

Genome sequencing and assembly of *I. pastinacae* isolate IP10 (Section 3.2.1, Table 3.1) was performed during this study. The assembled genome was used primarily to design *I. pastinacae* specific primers for PCR amplification and sequencing of housekeeping genes and selected functional genes. *I. pastinacae* isolate IP10 was subcultured from the leading edge of a culture onto MA and incubated at 20°C for five days to produce an actively growing colony. Three agar plugs of 5 mm were taken from the leading edge, placed into Petri dishes containing 25 mL PDB, and incubated at 20°C for 10 days. Agar plugs were removed and the mycelial mat washed three times in sterile RO water and blotted dry on tissue paper before being freeze dried overnight. Genomic DNA was extracted from the freeze-dried mycelium using DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. Isolate species identity was confirmed through PCR amplification and sequencing of the Internal Transcribed Spacer (*ITS*) region. PCR reactions consisted of 10 µl 1xREDTaq Ready Mix PCR reaction mix (Sigma-Aldrich UK), 2 µl DNA template (10 ng), 1 µl primer template (0.4 µmol L^{-1}) and 6 µl purified water (Sigma-Aldrich, UK). Specific annealing temperatures were used for each primer pair (Table 3.2). Following PCR reaction, amplicons were visualized using gel electrophoresis using 1.5% agarose (Fischer Scientific) gels in tris-acetate-EDTA (TAE) buffer, stained with 2 µl / 100 ml of GelRed (Biotium, UK). PCR purification was then performed to extract DNA from the PCR reaction mixture using the QIAquick PCR purification

Kit (Qiagen); 5 µl DNA template and 5 µl 5 pM sequencing primer (Table 3.2) were then submitted for sequencing by GATC (Konstanz, Germnay).

The genomic library was prepared using the Illumina TruSeq DNA library preparation kit following the manufacturers protocol (Essex, UK) and was sequenced on a HiSeq 2500 (Illumina) using a 51 bp paired end read. *De novo* assembly of the Itersonilia genome was built using SPAdes (Version 3.8.2) and a contig assembly file with an N50 value (the median length of 50% of the assembly contigs) of 1.6 kb was obtained.

3.2.7 Molecular characterisation of *Itersonilia* spp. isolates

Itersonilia spp. isolates were subcultured from the leading edge of a culture onto MA at 20°C for 10 days to produce actively growing colonies. Three 5 mm agar plugs were taken from the leading edge, placed into Petri dishes containing 25 mL PDB, and incubated at 20°C for 7 days. Agar plugs were then removed and the mycelial mat washed three times in sterile RO water and blotted dry on tissue paper before being freeze-dried overnight. Genomic DNA was extracted from the freeze-dried mycelium using the DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. Isolate species identity was confirmed through PCR amplification and sequencing of the *ITS* ribosomal DNA and Translation Elongation Factor 1-α (*EF1-*α), RNA polymerase II (*RpbII*), β-tubulin (*TUB2*), partial 18S ribosomal RNA gene (*SSU*) and partial 28S ribosomal RNA gene (*LSU*) to investigate intra-species diversity. Published universal fungal primers were used for amplifying the *ITS* and EF1-α regions, while primers for other housekeeping genes were specifically designed from the *I. pastinacae* whole genome sequence as described in Section 3.2.6.

Due to the lack of genetic variability within the housekeeping genes, additional functional genes were identified by comparing variable genes from annotated sequences of *Cryptococcus* spp. with the *I. pastinacae* genome sequence that we

had generated using BLAST analysis on NCBI. Four of these, triosephosphate transporter family (*TTF*), tRNA methyl transferase (*tMT*), cellobiose dehydrogenase (*CDH*) and sugar transporter genes (*STR*), were selected for amplification based on the predicted higher genetic variability to investigate intra-species diversity with PCR amplification carried out using specifically designed primers from the *I. pastinacae* genome sequence. PCR was carried out in 20 µl reactions using a range of primers (Table 3.2). PCR reactions consisted of 10 µl 1xREDTaq Ready Mix PCR reaction mix (Sigma-Aldrich UK), 2 µl DNA template (10 ng), 1 µl primer template (0.4 µmol L^{-1}) and 6 µl purified water (Sigma-Aldrich, UK). Specific annealing temperatures were used for each primer pair (Table 3.2). Full thermocycling conditions can be found in the appendix (Table A.1). Following PCR reaction, amplicons were visualized using gel electrophoresis using 1.5% agarose (Fischer Scientific) gels in tris-acetate-EDTA (TAE) buffer, stained with 2 µl / 100 ml of GelRed (Biotium, UK). PCR purification was then performed to extract DNA from the PCR reaction mixture using the QIAquick PCR purification Kit (Qiagen); 5 µl DNA template and 5 µl 5 pM sequencing primer (Table 3.2) were then submitted for sequencing by GATC (Konstanz, Germany).

Table 3.2 – Target gene loci, primers and annealing temperature used for PCR and sequencing of *Itersonilia* spp. isolates.

Genetic locus		Primer code	Primer sequence (5'-3')	Length (bp)	Annealing temp (°C)	Aplicon size (bp)	Source
ITS	Nuclear rDNA Internal transcribed spacer regions	ITS1	TCCGTAGGTGAACCTGCGG	19	61	444	Gardes & Bruns (1993)
		ITS4	TCCTCCGCTTATTGATATGC	20			White et al. (1990)
EF-1 α	Translation Elongation Factor 1- α	EF595F	CGTGACTTCATCAAGAACATG	21	61	392	Kauserud & Schumacher (2001)
		EF1160R	CCGATCTTGTAGACGTCCTG	20			
Rpb2	RNA Polymerase II Second largest subunit	IP RpbII F	GACTTTGACCTGACGCCCTCTC	22	68	1186	Designed from Isolate IP10 Genome
		IP RpbII R	AAGGGCCGAGATTCAGTCAG	20			
TUB2	Partial β-Tubulin	TUB2 55F	GCGTAGCCGACCATGAAGAAGC	22	68	559	Designed from Isolate IP10 Genome
		TUB2 536R	ACACGGTCGTCGAGCCCTACAA	21			
LSU	Partial 28s rRNA gene	IP LSU F	ATGCGAGTTTCTGCTATCCTGAG	23	59	214	Designed from Isolate IP10 Genome
		IP LSU R	ATCAATAAGCGGAGGAAAAGAAAC	23			
SSU	Partial 18s rRNA gene	IP SSU F	CGTCAATTCCTTTAAGTTTCAGC	23	48	821	Designed from Isolate IP10 Genome
		IP SSU R	TATCTGCCCTATCAACTTTC	20			
TTF	Triosephosphate Transporter Family	588 F	CCCCGGGCGCTGAGTAGG	18	70*	422	Designed from Isolate IP10 Genome
		1159 R	TGAGGGAGTGCGAGAAGTGTTAGC	24			
tMT	tRNA methyl transferase	36 F	GACGGGACCGATCTGCGACTGCTC	24	70*	612	Designed from Isolate IP10 Genome
		437 R	GCCGATGACCTGACGACCGCTGTG	24			
CDH	Cellobiose dehydrogenase	6381 F	GCAGTTGGCGCAGGCTATG	19	69*	610	Designed from Isolate IP10 Genome
		6927 R	AGGAGGCGTGAGAAGAGTGTGAGG	24			

3.2.8 Species specific primer design for *Itersonilia* spp.

Primers designed for the amplification of functional genes as previously described (Section 3.2.6); triosephosphate transporter family, tRNA methyl transferase, cellobiose dehydrogenase and sugar transporter were tested for specificity to *Itersonilia* spp. Primers were tested against a range of soil and seed-borne fungal pathogens (Table 3.4), to determine specificity to *Itersonilia* spp. Genomic DNA from fungal pathogens was extracted as described above (Section 3.2.7), from freeze-dried mycelium using the DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. PCR was carried out as 20 µl reactions using primers designed from the *I. pastinacae* genome sequence (Section 3.2.7, Table 3.2), and amplified using thermocycling conditions as previously described (Section 3.2.7), modified to identify optimum conditions. Following amplification, PCR amplicons were visualised using 1.5% agarose (Fischer Scientific) gel in tris-acetate-EDTA buffer, stained with 2 µl / 100 ml of GelRed (Biotium, UK).

Table 3.3 – *Itersonilia* spp. specific primers and target gene loci used in PCR amplifications

Gene	Primer code	Sequence (5' - 3')	Annealing temp (°C)	Amplicon size (bp)
Triosephosphate Transporter Family	588 F	CCCCGGGCGCTGAGTAGG	70	571
	1159 R	TGAGGGAGTGCGAGAAGTGTTAGC		
tRNA methyl transferase	36 F	GACGGGACCGATCTGCGACTGCTC	70	401
	437 R	GCCGATGACCTGACGACCGCTGTG		
Cellobiose Dehydrogenase	6381 F	GCAGTTGGCGCAGGCTATG	69	546
	6927 R	AGGAGGCGTGAGAAGAGTGTGAGG		
Sugar Transporter	58 F	GAGGCCACCGACGACCCATCT	68	1142
	1200 R	GAGGCTCCTACTTCTAGTCCCG		

Table 3.4 – Table of seed and soil-borne fungal species for testing against *Itersonilia* spp. specific primers. Species name, host and origin are detailed.

Number	Species	Host	Origin
1	*Fusarium oxysporum*	Bean	USA
2	*F. oxysporum*	Pea	UK
3	*F. oxysporum*	Flax	UK
4	*F. oxysporum*	Carnation	UK
5	*F. oxysporum*	Daffodil	UK
6	*F. oxysporum*	Freesia	Netherlands
7	*F. oxysporum*	Banana	UK
8	*F. oxysporum*	Leek	UK
9	*F. oxysporum mathiola*	Stocks	UK
10	*F. oxysporum gladioli*	Gladioli	UK
11	*F. oxysporum vasinfectum*	Cotton	UK
12	*F. oxysporum melonis*	Melon	UK
13	*F. oxysporum conglutinanas (Race 2)*	Brassica	UK
14	*F. oxysporum radicis-lycopersici*	Tomato	UK
15	*F. oxysporum lycopersici (Race 1)*	Tomato	UK
16	*F. oxysporum*	Rocket	UK
17	*Fusarium equiseti*	Rocket	UK
18	*Fusarium redolens*	Rocket	UK
19	*Fusarium avanaceum*	Leek	UK
20	*Fusarium proliferatum*	Onion	UK
21	*Fusarium culmorum*	Leek	UK
22	*Fusarium fujiluroi*	Banana	UK
23	*Fusarium saccarhi*	Banana	UK
24	*Fusarium trinctum*	Brassica	UK
25	*Fusarium poae*	Pea	UK
26	*Fusarium solani*	Pea	UK
27	*Fusarium pseudocircinatum*	Banana	UK
28	*Fusarium graminerum*	Onion	UK
29	*Botrytis aclada*	Onion Seed	UK
30	*Botrytis allii*	Onion Seed	UK
31	*Botrytis cinerea*	Onion Seed	UK
32	*Plectosphaerella cucumerina*	Brassica	UK

Number	Species	Host	Origin
33	*Neonectria sp.*	Snowdrop	UK
34	*Trichoderma sp.*	Onion	UK
35	*Cylindrocarpon destructans*	Parsnip Root *	UK
36	*Sclerotinia sclerotiorum*	Pea	UK
37	*Pythium ultimum*	Carrot	UK
38	*Pythium violae*	Carrot	UK
39	*Mycocentrospora acerina*	Parsnip Root *	UK
40	*Itersonilia pastinacae*	Parsnip Seed *	UK
41	*Sclerotium cepivorum*	Onion	UK
42	*Phoma* sp.	Onion	UK
43	*Rhizoctonia solani*	Unknown	UK
44	*Verticillium albo'atrum*	Potato	UK
45	*Alternaria alternata*	Parsnip seed (cv. Panache) *	UK
46	*Cladosporium tenuissimum*	Parsnip seed (cv. Panache) *	UK
47	*Penicillium chrysogenum*	Parsnip seed (cv. Panache) *	UK
48	*Alternaria* sp.	Parsnip seed (cv. Panache) *	UK
49	*Phoma fungicola*	Parsnip seed (cv. Panache) *	UK
50	*Alternaria* sp.	Parsnip seed (cv. Panache) *	UK
51	*Trichoderma hamatum*	Parsnip seed (cv. Panache) *	UK
52	*Cladosporium cladosporoides*	Parsnip seed (cv. Panache) *	UK
53	*Cladosporium cladosporoides*	Parsnip seed (cv. Panache) *	UK
54	*Penicillum olsonii*	Parsnip seed (cv. Panache) *	UK
55	*Alternaria infectoria*	Parsnip seed (cv. Panache) *	UK
56	*Alternaria infectoria*	Parsnip seed (cv. Panache) *	UK
57	*Fusarium chlamydosporum*	Parsnip seed (cv. Panache) *	UK
58	*Sporobolomyces ruberrius*	Parsnip seed (cv. Panache) *	UK

* Indicates fungal pathogens isolated in this project; remaining isolates were collected in other research projects.

3.2.9 Transcriptome sequencing, assembly and gene expression analysis of *I. pastinacae* fungal and yeast phases

Whole transcriptome sequencing and assembly was carried out on *I. pastinacae* isolate IP10 (Section 3.2.1, Table 3.1) during both the fungal and yeast stage of the pathogen. Isolate IP10 was subcultured from the leading edge of an actively growing colony onto MA at 20°C for 10 days to produce an actively growing colony. An agar plug of 5 mm was placed in the centre of a Petri dish of 25 ml MA covered in a 0.2 µl nylon membrane filter (Whatman, England). Plates were sealed with parafilm and incubated at 20°C for 10 days for development of the fungal stage, and 20 days for development of the yeast stage of the pathogen. The mycelial mat was removed from the nylon filter, wrapped in sterile foil and rapidly frozen by placing in liquid nitrogen for three minutes, then stored at -80°C. RNA was extracted from the frozen mycelium using the RNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturers protocol; DNA was removed using DNase I (Sigma-Aldrich). RNA purity was confirmed by running samples on a Bioanalyser (Agilent), and two libraries were prepared using the Illumina TruSeq RNA kit following the manufacturers protocol. These were pooled and run on the Illumina MiSeq using a 200 bp paired end run with the V2 cartridge kit (Illumina). *De novo* transcriptome assembly was carried out using the programme Trinity.

For the gene expression analysis, genes were predicted in the contigs from the Itersonilia genome de novo assembly (Section 3.2.8) using AUGUSTUS (Version 3.2.2) from which a gtf file was prepared to identify the start and stop positions of genes, and exons, in each contig. The RNA-seq reads for both the fungal- and yeast- form samples were cleaned using Trimmomatic for adapter sequences. These were then mapped to the earlier prepared assembly using STAR (Version 2.5.2), from which a gtf file was produced to the number of reads mapped to genes. Finally, differentially expressed genes were found using the DESeq2 programme, with the most significant ones filtered out, and selecting the genes with at least abs(log2foldchange) ≥ 1, and p-adjusted (BH, Benjamini,- Hochberg

procedure) of ≤ 0.1. A fasta file of the gene sequences was extracted from the contig assembly with a blastx search providing the annotations of each gene.

3.2.10 Phylogenetic analysis of housekeeping and functional gene sequence data for *Itersonilia* spp. isolates

Housekeeping and functional gene sequences obtained for all *Itersonilia* spp. Isolates were trimmed and aligned using the ClustalW algorithm and refined using the MUSCLE algorithm implemented in MEGA v6 (Tamura *et al.*, 2013). Two multi-locus concatenated phylogenetic trees for each species was drawn, one using the 'Maximum Likelihood' and one the 'Neighbour Joining' option, rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005). .

3.2.11 Statistical analysis and modelling of temperature effects on growth of *Itersonilia* spp. isolates.

The size of the *Itersonilia* spp. colonies was measured twice weekly for 30 days and, by visual inspection the period between day four and day eleven was selected as the optimum for calculation of the growth rate (mm day^{-1}) and further analysis (by day four, isolates had a consistent growth rate, whilst at day 11 maximum colony size had not yet been reached at any temperature). Data was analysed using a non-linear regression of biological temperature-dependent rate, based on absolute reaction-rate theory by Schoolfield *et al.* (1981). This model is derived based upon the assumptions that: 1) at all temperatures, the developments rate of a poikilotherm population is determined by a single rate-controlling enzyme reaction and 2) this rate-controlling enzyme is reversibly denatured at high and low temperatures but maintains a constant total concentration independent of temperature. The model is derived from that of Sharpe & DeMichele (1977), who describe how the rate of a biological process is affected by temperature. Schoolfield *et al.* (1981) noted how the original model of Sharpe and DeMichele (1977) is ill-suited for linear regression techniques due

to the high correlation between model parameters, so re-parameterised it in the following form:

$$r(T) = \frac{\rho_{(25°C)}\ \frac{T}{298}\exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2_L}} - \frac{1}{T}\right)\right] + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}, \quad (1)$$

where $r(T)$ is the mean growth rate at temperature T (time^{-1}), T is temperature in °K (298°K = 25°C), and R is the universal gas constant (1.987 cal deg^{-1} mol^{-1}). The other parameters are associated with the rate controlling enzyme reaction: $\rho_{(25°C)}$ is the development rate at 25°C assuming no enzyme inactivation (time^{-1}), $\Delta H_A^{\neq}$ is the enthalpy of inactivation of the reaction that is catalysed by the enzyme (cal mol^{-1}), $T_{1/2_L}$ is the temperature (°K) at which $\frac{1}{2}$ the enzyme is active and $\frac{1}{2}$ is inactive due to low temperatures, ΔH_L is the change in enthalpy associated with low temperature inactivation of the enzyme (cal mol^{-1}), $T_{1/2_H}$ is the temperature (°K) at which the enzyme is $\frac{1}{2}$ active and $\frac{1}{2}$ high temperature inactive, ΔH_H is the change in enthalpy associated with high temperature inactivation of the enzyme (cal mol^{-1}). A reference temperature of 25°C was chosen by Schoolfield *et al.* (1981), although they mention other temperatures would have been equally valid and significantly different temperatures might need to be selected for other organisms.

In order to perform the non-linear regression using equation (1), suitable starting values for the parameters were chosen following the suggestion of Schoolfield *et al.* (1981) and Davidson *et al.* (2003), i.e. by identifying the slopes of straight line regions of a curve fitted by eye to available data on an Arrhenius plot. Based on the data, in particular from IP10, for which growth rate at additional temperatures was measured, the intermediate straight portion of the fitting curve on an Arrhenius plot suggests 15°C as a more reasonable reference temperature, rather than the 25 °C used in (1). Therefore, the following modified form of equation (1) was used:

$$r(T) = \frac{\rho_{(15°C)}\ \frac{T}{288}\exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{288} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2_L}} - \frac{1}{T}\right)\right] + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}. \quad (2)$$

When the data does not show evidence of reduced growth due to either high or low temperature inactivation, Schoolfield *et al.* (1981) suggest removing the exponential term of the denominator describing the corresponding reduction in growth. This eliminates two out of the six parameters that the data would be unable to identify. Given the data collected in this study always show evidence of inactivation due to high temperatures but not always due to low temperatures, and given our choice of reference temperature of 15 °C, the four parameter reduced model used here takes the form:

$$r(T) = \frac{\rho_{(15°C)}\ \frac{T}{288}\exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{288} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}, \quad (3)$$

where parameters ΔH_L and $T_{1/2_L}$ have been removed.

Growth rates were calculated for all *Itersonilia* spp. isolates at six temperatures (0, 5, 10, 15, 20 and 25°C); using these data alone it was not always possible to estimate six different parameters and therefore the four-parameter model (3) was used. However, the additional measurements collected for the standard *Itersonilia* spp. isolate, IP10, were sufficient to estimate six independent parameters. Model fitting was carried out in MATLAB (Version 2015b) using the nlinfit function for non-linear regression curve fitting. Statistical analysis of growth rates for *Itersonilia* spp. isolates used a one-way ANOVA to determine isolate as a significant factor. A posthoc 'Tukey' test was carried out for each temperature (0, 5, 10, 15, 20 and 25°C) to identify differences between isolates.

3.2.12 Statistical Analyses of *Itersonilia* spp. pathogenicity assays and spore production experiments

All statistical analysis was carried out using the software R (version 0.98.945, R Development Core Team, 2014). For data from the *Itersonilia* spp. pathogenicity experiments on parsnip roots, lesion area (cm^2) was analysed using a one-way ANOVA; no further posthoc test was required. From the *Itersonilia* spp. pathogenicity experiments on parsnip leaves, lesion areas (cm^2) were log_e-transformed to satisfy the requirements of homogeneity of variance and reduce the influence of residuals, and analysed using a one-way ANOVA with the 5% LSD value calculated to directly compare isolates. For experiments examining both chlamydospore and ballistospore production by *Itersonilia* spp., mean log_{10} spore count density (spores mm^{-2}) for each spore type was plotted against temperature. Data were then analysed using a one-way ANOVA, and the posthoc test 'Tukey' applied to determine differences between isolates at each temperature.

3.3 Results

3.3.1 Pathogenicity of *Itersonilia* spp. isolates on parsnip roots

Following inoculation with *Itersonilia* spp. isolates, all parsnip roots developed lesions (Fig 3.1), with mean lesion area ranging from 2.2 cm^2 to 3.2 cm^2 after 60 days (Fig 3.2). There was a continuum of lesion area across the *Itersonilia* spp. isolates, but no groups or outliers were identified within the dataset. The ANOVA of the lesion area (cm^2) indicated no significant effect of *Itersonilia* spp. isolate on lesion size ($p>0.05$). *Itersonilia* spp. isolates from all hosts were evenly distributed, with no detectable correlation between host and lesion area.

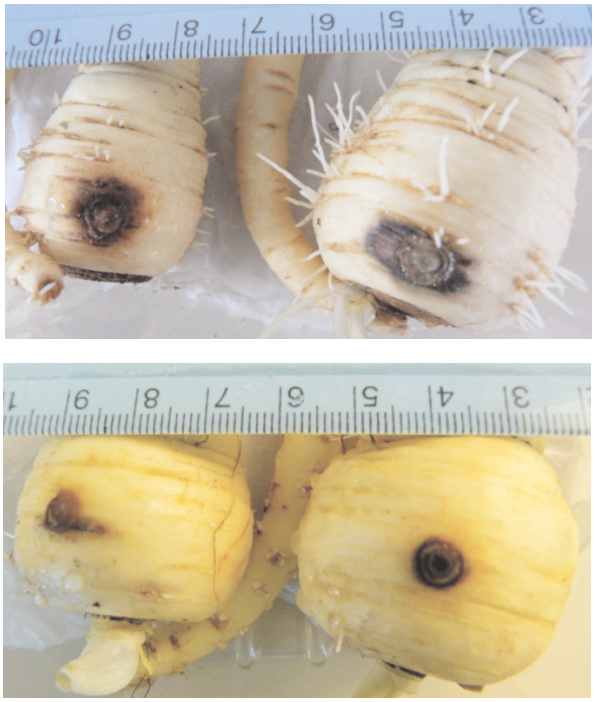


Figure 3.1 – Symptoms of *Itersonilia* spp. on inoculated parsnip roots (cv. Picador) after 21 days at 20°C. Top; highly aggressive *Itersonilia spp.* isolate IP50; less aggressive *Itersonilia* spp. isolate IP15.

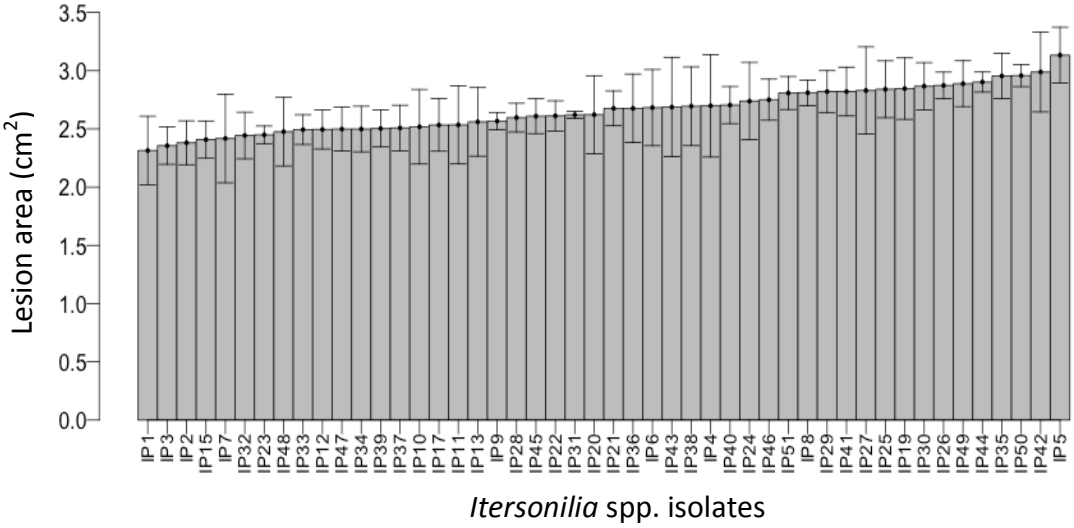


Figure 3.2 – Lesion area (cm^2) on parsnip roots (cv. Picador) inoculated with 48 different *Itersonilia* spp*.* isolates. Error bars are SEM from four independent replicates.

3.3.2 Pathogenicity of *Itersonilia* spp*.* isolates on detached parsnip leaves

Following inoculation, all 48 *Itersonilia* spp*.* isolates caused necrotic lesions on detached parsnip leaves (cv. Panache) (Fig 3.3), with the mean lesion area ranging from 0.04 cm^2 (IP7) to 0.3 cm^2 (IP24) (corresponding to log_e values ranging from -3.0 to -0.2 in Fig 3.4). Log_e-transformed data was analysed using a one-way ANOVA, which showed a significant effect of isolate ($P<0.05$) on lesion size. Whilst *Itersonilia* spp*.* isolates from different hosts were evenly distributed with no correlation between host and lesion area. The posthoc 5% LSD values indicated that IP7 and IP24 were significantly different ($p<0.05$) from all others (Table 3.5). Isolates IP25 and IP48 also appear as significantly different from the majority of other isolates ($p<0.05$), with larger lesion sizes. Due to the variable SEM observed in many of the other *Itersonilia* spp*.* isolates, the majority of isolates ranked in the middle were only significantly different ($p<0.05$) to isolates displaying extreme (higher or lower) lesion areas.

In comparing the virulence of isolates from both the parsnip root and leaf pathogenicity assays there is little correlation. Isolates displaying higher

virulence on parsnip leaves show varying levels of high and low virulence on parsnip root. Similarly isolates displaying high virulence on parsnip root display varying levels of virulence on parsnip leaves suggesting virulence may be tissue specific.

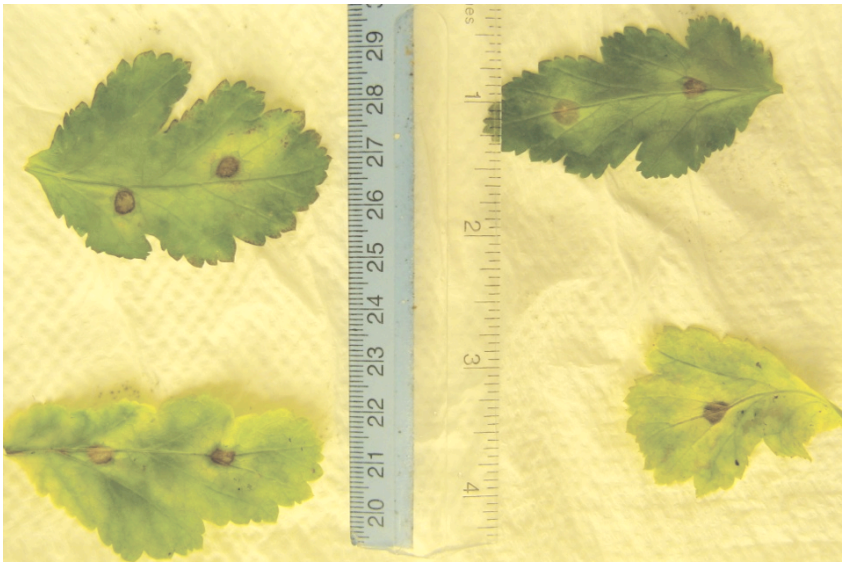


Figure 3.3 – Necrotic symptoms on parsnip leaves (cv. Panache) 7 days after inoculation with 20 µl drop of 1 x 10^5 *I. pastinacae* isolate IP10.

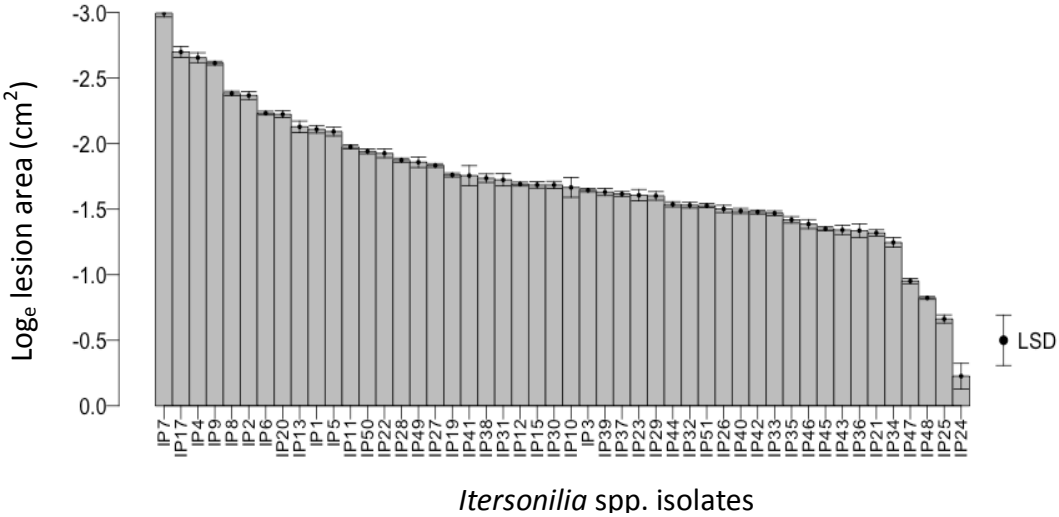


Figure 3.4 – Log$_e$ transformed lesion area (cm^2) on detached parsnip leaves (cv. Panache) inoculated with 48 different *Itersonilia* spp. isolates. Error bars are SEM from four independent replicates.

Table 3.5 – Summary of log_e transformed means and the 5% LSD value for all *Itersonilia* spp. isolates from parsnip leaf pathogenicity assays. The degrees of freedom (d.f.) were 47.

Number	Isolate	Log_e transformed mean	5% LSD
1	IP24	-0.1479802	a
2	IP25	-0.6275184	b
3	IP48	-0.782651	b
4	IP47	-0.905793	bc
5	IP34	-1.1956396	cd
6	IP21	-1.2926864	de
7	IP36	-1.3003438	def
8	IP43	-1.3203833	defg
9	IP46	-1.3421869	defg
10	IP45	-1.3439921	defg
11	IP35	-1.401353	defgh
12	IP33	-1.4406046	defghi
13	IP42	-1.4692718	defghi
14	IP40	-1.4731119	defghi
15	IP26	-1.4884654	defghij
16	IP32	-1.4994631	defghij
17	IP51	-1.5186464	defghijk
18	IP44	-1.5234973	defghijk
19	IP29	-1.569913	defghijkl
20	IP39	-1.5723161	defghijklm
21	IP23	-1.5826685	efghijklm
22	IP10	-1.5829393	efghijklm
23	IP37	-1.5910244	efghijklm
24	IP3	-1.6362592	efghijklm
25	IP30	-1.6693541	efghijklmn
26	IP15	-1.6754654	efghijklmn
27	IP12	-1.6835767	fghijklmn
28	IP38	-1.6839003	fghijklmn
29	IP31	-1.6845307	fghijklmn
30	IP41	-1.6860966	ghijklmn
31	IP19	-1.7503632	hijklmno
32	IP49	-1.8024137	jklmno
33	IP27	-1.8102314	ijklmnop
34	IP28	-1.860816	jklmnop
35	IP22	-1.8991831	klmnopq

36	IP50	-1.9270011	lmnopq
37	IP11	-1.9549057	mnopqr
38	IP5	-2.0455927	nopqrs
39	IP1	-2.0803795	opqrs
40	IP13	-2.1022134	opqrs
41	IP20	-2.1875356	pqrs
42	IP6	-2.2174032	qrst
43	IP2	-2.3393048	rstu
44	IP8	-2.3499942	stu
45	IP4	-2.5944395	tuv
46	IP9	-2.6007393	tuv
47	IP17	-2.6583638	uv
48	IP7	-2.9230709	v
d.f.	**47**		
5% LSD	**-0.3847903**		

3.3.3 Effect of temperature on *Itersonilia* spp. growth rate

All *Itersonilia* spp. isolates grew at all six temperatures tested (0, 5, 10, 15, 20 and 25°C) with a general trend of increasing growth rate with increasing temperature over the range (Fig 3.5). The minimum average rate of growth over all isolates was at 0°C (0.48 mm day^{-1}), with values ranging from 0.38 to 0.59 mm day^{-1}, and the maximum average growth rate at 25°C (3.66 mm day^{-1}), with values ranging from 1.07 to 4.43 mm day^{-1}. The growth rate at 0°C displayed the smallest variation between isolates, with isolate growth rates at 5°C displaying the greatest variation (from 0.05 to 2.66 mm day^{-1}).

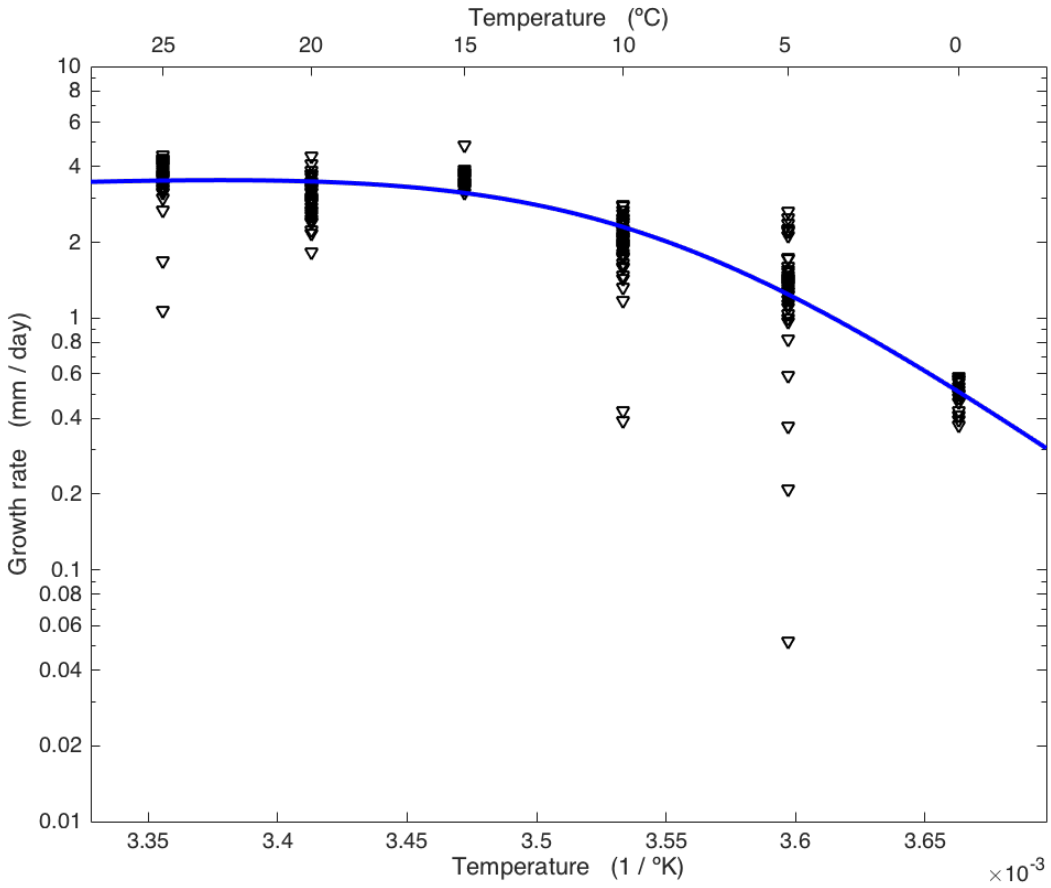


Figure 3.5 – Model predictions for the effect of temperature on the growth rate of *I. pastinacae* isolates (Table 3.1). Blue line represents fitted Arrhenius curve obtained using equation (3) for *I. pastinacae* isolates grown on MA, with black triangles representing the mean growth rate over four replicates for individual isolates.

Some *I. pastinacae* isolates such as IP11, IP17, IP26 and IP45, displayed consistently lower growth rates compared to other isolates at most temperatures, whilst isolates IP4, IP34, IP37 and IP46 displayed consistently higher growth. Some isolates such as IP38 and IP44 appeared to grow preferentially at high temperatures whilst others including IP2, IP6 and IP30 appeared to grow preferentially at low temperatures. *I. pastinacae* isolate IP10 (standard genome sequenced isolate) was ranked consistently in the middle range of growth rates.

The ANOVA revealed that *I. pastinacae* isolate had a significant effect on growth rate. A posthoc 'Tukey' analysis carried out to perform pairwise comparisons

between isolates showed that the largest number of significant differences between isolates was at 5°C, where 10 *I. pastinacae* isolates were each significantly different ($p<0.001$) from at least 34 other isolates, with IP11, IP17, IP36 and IP38 displaying the lowest growth rate, and IP1, IP2, IP4, IP20, IP21 and IP24 displaying the highest growth rate. At 10°C, IP11 and IP17 (the two low growth rate outliers at 10°C in Fig 3.5) are significantly different ($p<0.001$) from all other isolates. Significant differences ($p<0.05$) at this temperature were also detected between isolates with low growth rates (IP38, IP44, IP45) or high growth rate (IP34, IP37, IP40 and IP41) and the remaining isolates in the middle (see Table A.2 in the appendix). At 15°C, IP9 (the high growth-rate outlier at 15°C in Fig 5.3) was significantly different ($p<0.001$) from all other isolates. Isolates IP5, IP6 and IP45, with the lowest growth rates, also showed some significant difference ($p<0.05$) from other isolates. At 20°C, the isolates significantly different from most others were IP11 (low growth rate, Fig 3.5) and IP10, IP13 and IP34 (high growth rate). At 25°C, isolates IP11 and IP17 were significantly different from almost all other isolates (low growth rate outliers, Fig 3.5). The full details of the 'Tukey' analysis for comparison between all isolates at all temperatures can be found in the appendix (Table A.2).

In addition to the six temperatures discussed above, IP10 was selected as a standard isolate for *I. pastinacae* and assessed across further five temperatures of 2.5, 17.5, 22.5, 27.5 and 30°C (Fig 3.6). This isolate displayed the lowest average rate of growth at 0°C (0.5 mm/day), with no growth at 30°C, and maximum rate of growth between 20 and 22.5°C (4.11 and 3.71 mm day^{-1}, respectively). Data for these additional temperatures allowed accurate fitting of the six-parameter model of equation (2).

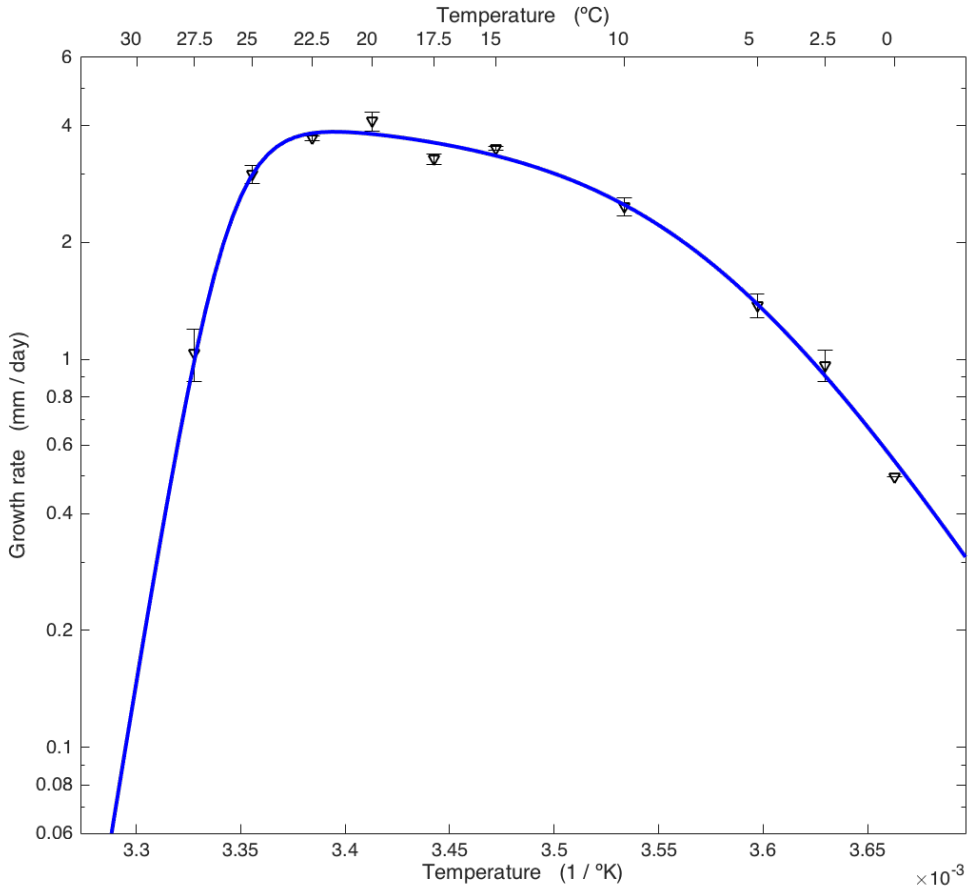


Figure 3.6 – Model predictions for the effect of temperature on the growth rate of *I. pastinacae* isolate IP10 (Table 3.1) on an Arrhenius plot. Blue line represents fitted curve obtained using equation (2) for IP10 grown on MA, with black triangles representing mean growth rate of four replicates; error bars show SEM.

The parameter estimates from the model fitting (Table 3.6) vary significantly between the four parameter model (equation (3)) fitted to all the *Itersonilia* spp. isolates and the six parameter model (equation (2)) fitted to the standard *I. pastinacae* isolate, IP10. As an example, $\rho_{(15°C)}$, the estimated rate of growth at 15°C (assuming no enzyme inactivation), is 13.65 mm day^{-1} for all the *Itersonilia* spp. isolates and is biologically less reasonable than the 3.88 mm day^{-1} estimated for *I. pastinacae* isolate IP10. These differences can be attributed to two main reasons. First, the growth rate of *I. pastinacae* isolate IP10 was measured at 0°C and 30°C. At these temperatures, very low growth rates were recorded in contrast to the higher growth rates at intermediate temperatures. This enabled

better estimates for the temperatures at which half high enzyme inactivation and half low enzyme inactivation occur, leading to biologically plausible parameter estimates. Second, from the ANOVA analysis described above, significant differences were identified between isolates at all temperatures. This made it challenging for the four-parameter model to accurately capture such a high level of variation.

Table 3.6 – Summary of parameter estimates for the effect of temperature on the growth rates of colonies of *Itersonilia* spp. isolates and standard *I. pastinacae* isolate IP10. Estimation based on the Schoolfield et al. (1981) equations (2) and (3), modified for a reference temperature of 15°C as described in the text.

	$\rho_{(15°C)}$ mm day^{-1}	$\Delta H_A^{\neq}$ cal mol^{-1}	ΔH_H cal mol^{-1}	$T_{1/2_H}$ °C	ΔH_L cal mol^{-1}	$T_{1/2_L}$ °C
Itersonilia spp. isolates	13.615	32517	34989	9.4386	-	-
I. pastinacae isolate IP10	3.8778	690.71	1.55×10^5	26.127	-36541	6.9356

3.3.4 Effect of temperature on *Itersonilia* spp. spore production

The number of chlamydospores and ballistospores produced by *Itersonilia* spp. isolates was assessed over six temperatures (0, 5, 10, 15, 20 and 25°C), with the total spore production for each spore type expressed as spore density (spores mm^{-2}), to control for the variation in colony size observed at different temperatures. The general trend of the data suggested that chlamydospore production was moderately higher than ballistospore production at low temperatures and significantly lower than ballistospore production at high temperatures (Fig 3.7). Ballistospore density ranged from 289 to 1,923 spores mm^{-2} across all temperatures, and showed a similarly high range of values at 0°C and 20°C, with 1083 – 1922 spore mm-^{2} and 567 – 1513 spores mm-^{2} respectively (Fig. 3.7). The highest average chlamydospore density was at 0°C, with values ranging from 1,975 (IP1) to 2,451 (IP38) spores mm^{-2}, and decreased steadily with increasing temperature (Fig. 3.7). Whilst the lowest average

chlamydospores density across all isolates was at 20°C, where values ranged from 17.6 (IP10) to 64.27 (IP21) spores mm^{-2}. *I. pastinacae* isolates IP11 and IP17 did not produce any spores at any temperature so were removed from the graph and subsequent analysis.

ANOVA analysis showed a significant effect of *I. pastinacae* isolate ($p<0.05$) on spore production, for both ballistospores and chlamydospores; the post-hoc 'Tukey' analysis was then performed to identify significant differences between isolates at each temperature. There were no significant differences between isolates when analysing chlamydospore production, but significant differences ($p<0.05$) between isolates when analysing ballistospore production at 0, 10, 15, 20 and 25°C. The results showed an increase in significant differences between isolates ($p<0.05$) as temperature increased, with *I. pastinacae* isolates IP4, IP6, IP22 displaying consistently low densities while others, such as IP29, IP32 and IP38 displaying consistently high densities for both ballistospores and chlamydospores across most temperatures. Full results of the 'Tukey' analysis can be found in the appendix (Table A.3).

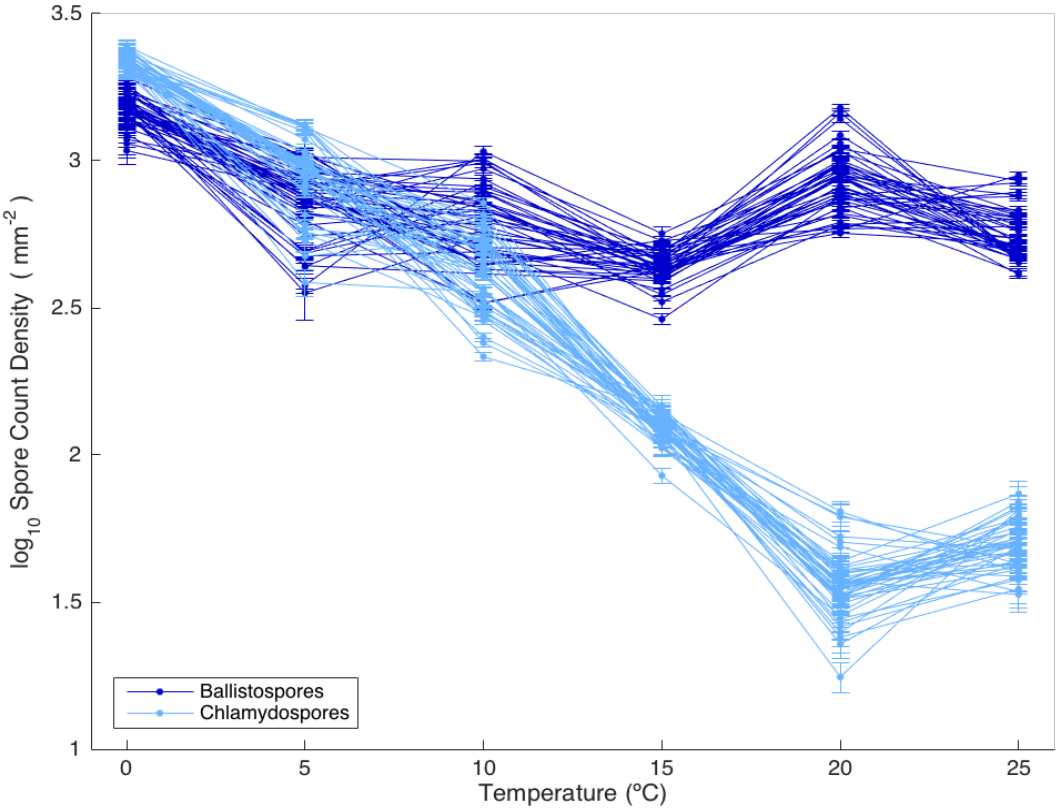


Figure 3.7 – Effect of temperature on Log_{10} spore density (spores mm^{-2}) for different *Itersonilia* spp. isolates (Table 3.1) grown on MA for 12 days. Data-points represent mean spore density of four replicates, error bars show SEM.

3.3.5 Molecular characterisation of *Itersonilia* spp. isolates

Following confirmation of Itersonilia species identity through *ITS* sequencing and BLAST analysis, sequence data for *ITS*, for the housekeeping genes *EF-1α*, *Rpb-II*, *LSU*, *SSU* and *TUB2* and for the three functional genes triosephosphate transporter family, tRNA methyl transferase and cellobiose dehydrogenase was assembled and a phylogenetic analysis was carried out to identify intra-species diversity.

The maximum likelihood and neighbour joining analyses for the *ITS* locus separated the *Itersonilia* isolates into two major clades (I and II), of 48 and two isolates respectively (Fig 3.8 and Fig 3.9). No variation was seen in the clade containing the two isolates but the clade with 48 isolates was further divided

into three clusters, none of which was related to host species or geographic location. For example, isolates with the same sequence were found from Lincolnshire, Scotland, Nottingham and Middlesex, and isolated from parsnip, dill, chrysanthemum and fennel. The two isolates comprising the other major clade (IP38 and IP44), were isolated from parsley from Middlesex and parsnip seed from France respectively, again suggesting that clustering is not related to host and location. Bootstrap values on both the maximum likelihood and neighbour joining analyses were all above 60% with the majority above 90%, indicating good support for clades.

Concatenated alignments of the housekeeping genes; *ITS*, *EF-1α*, *Rpb-II*, *LSU*, *SSU* and *TUB2* were analysed using both maximum likelihood and neighbour joining analyses (Fig 3.10 and Fig 3.11). Both phylogenies were very similar and divided the *I. pastinacae* isolates into six clades with each clade further sub-divided into different genetic clusters. Each of these smaller clusters contained between 1 and 18 isolates. None of the isolates within these genetic clusters appeared to be related by host or geographical location, with isolates in a single cluster being isolated from a range of host species and locations. The isolates separated visually into four groups, I, II, III and IV. Group I consisted of isolates from parsnip, chrysanthemum, dill, fennel and parsley from a range of locations, II contained isolates from parsnip and chrysanthemum from various locations, III contained isolates from parsnip hosts only from a range of locations and IV contained isolates from parsnip only from the maximum likelihood analysis and parsnip and dill from the neighbour joining analysis, in both cases from various locations. The bootstrap values for both the maximum likelihood and neighbour joining analysis range from 40-100%, with the majority of branches between 86-100%; these high values indicate good support for branches.

Maximum likelihood and neighbour joining phylogenies were constructed from concatenated alignments of the three functional genes: triosephosphate transporter family, tRNA methyl transferase and cellobiose dehydrogenase (Fig

3.12 and Fig 3.13). Both analyses divided the *I. pastinacae* isolates into six clades, with each clade further sub-divided into further genetic clusters. Each of the smaller clusters contained between 1 and 7 isolates, none of which appeared to be linked to host or geographical location. The clusters separated visually into five group: I, II, III, IV and V. The maximum likelihood analysis placed isolates from parsnip hosts only, from a range of locations into groups I and V. Group II contained isolates from parsnip, dill and parsley, whilst group III contained isolates from parsnip, chrysanthemum and dill and group IV contained isolates from parsnip and fennel; all groups contained isolates from a range of locations. The neighbour joining analysis, grouped the majority of isolates similarly, however, there were some differences: groups II and IV contains isolates from parsnip hosts only, group I contained isolates from parsnip, chrysanthemum and dill, group III contained isolates from parsnip, dill and parsley and group V contained isolates from parsnip and fennel. Again all isolates in each group were from a range of locations. This suggested genetic clusters were not grouped based on host species or geographical origin. The bootstrap values range from 30-100% across both the maximum likelihood and neighbour joining analyses, however the majority of branches display values greater than 80%, indicating good support for branches.

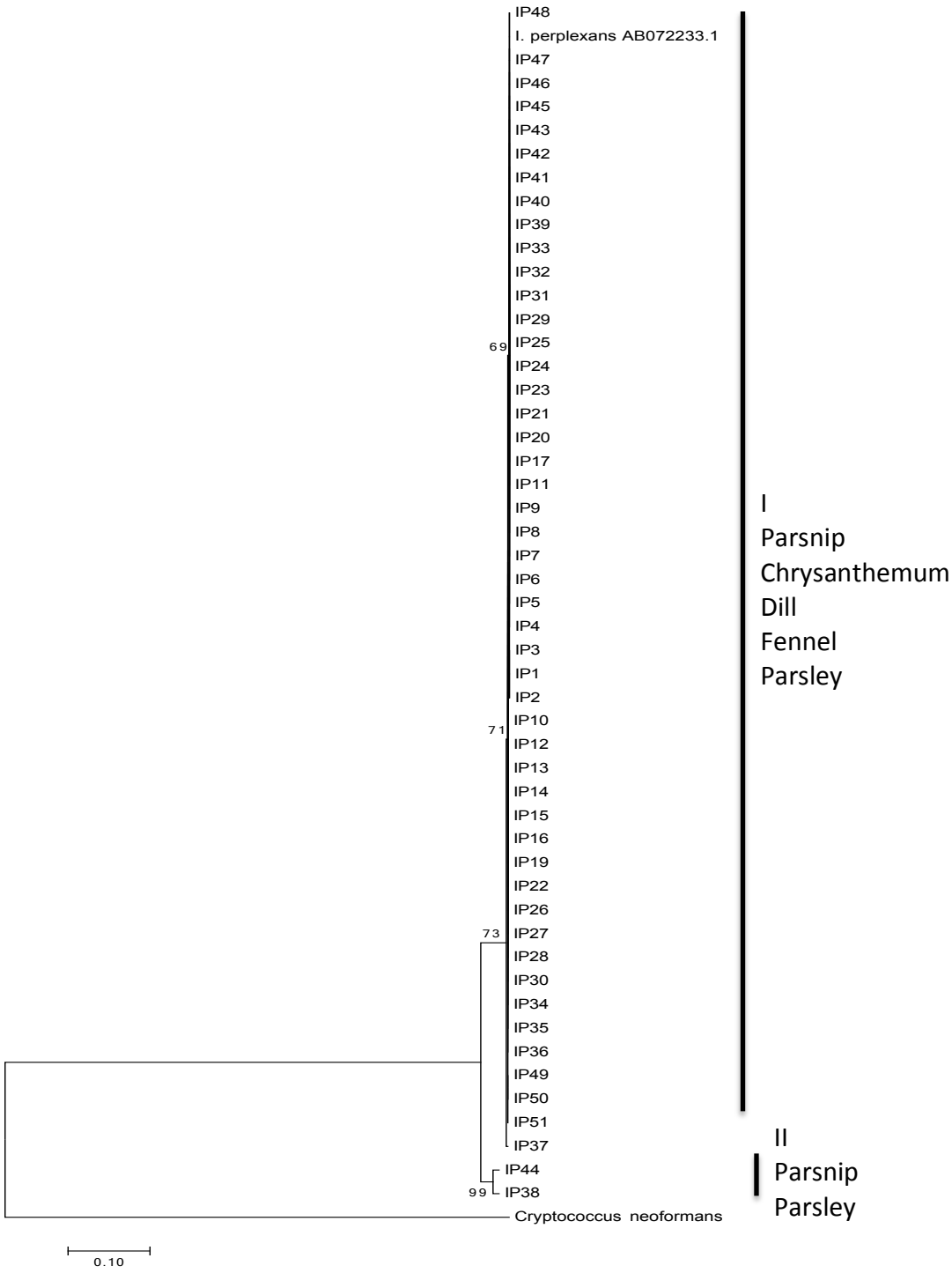


Figure 3.8 – Maximum Likelihood phylogenetic tree for *Itersonilia* spp. isolates based on the internal transcribed spacer (*ITS*). Numbers represent bootstrap values from 1,000 values. Scale bar indicates 0.10 substitutions per site. The tree is rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005). *I. perplexans* reference isolate (AB072233.1) is also included.

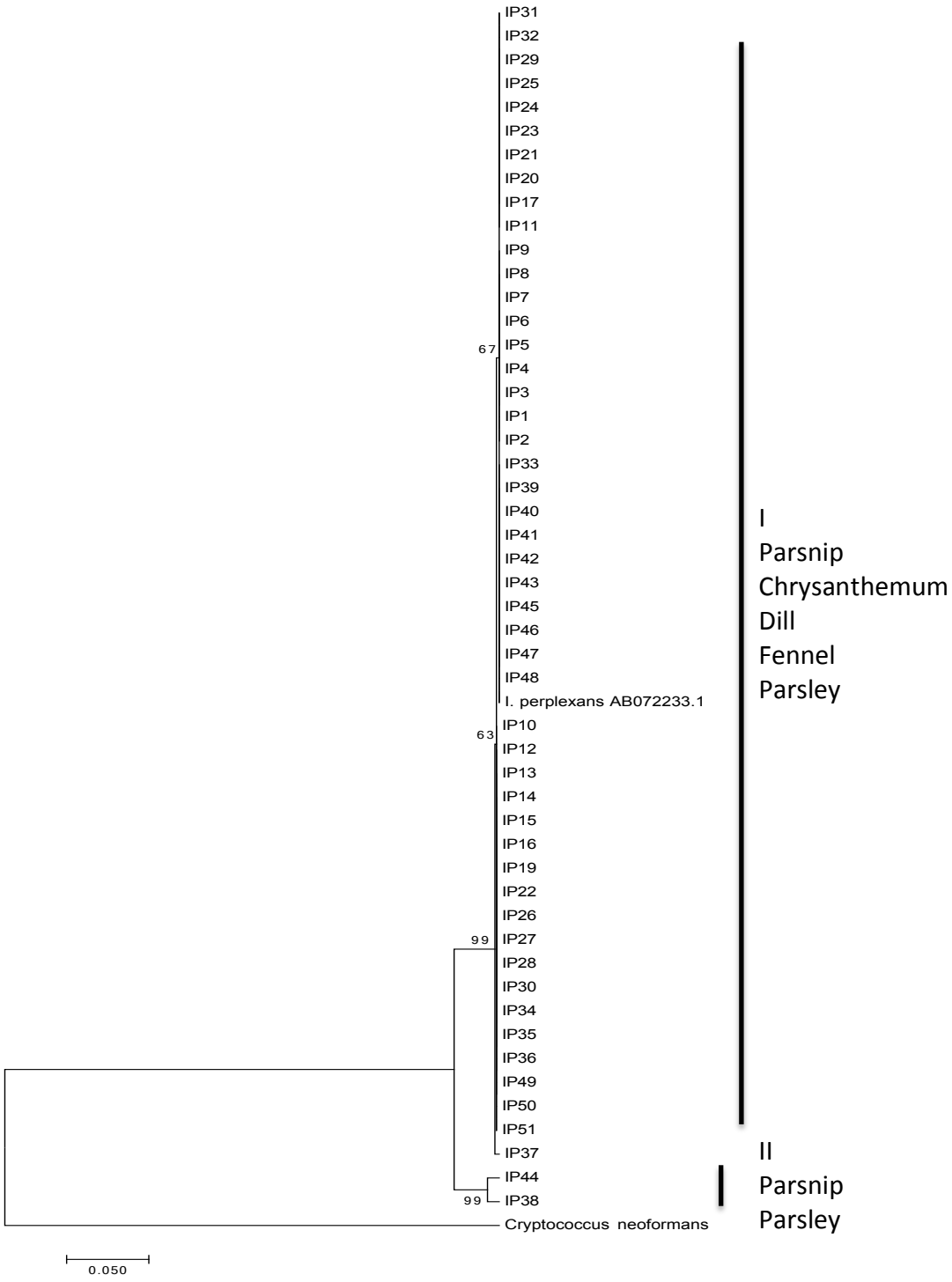


Figure 3.9 – Neighbour Joining phylogenetic tree of *Itersonilia* spp. isolates based on the internal transcribed spacer (*ITS*). Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site. The tree is rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005). *I. perplexans* reference isolate (AB072233.1) is also included.

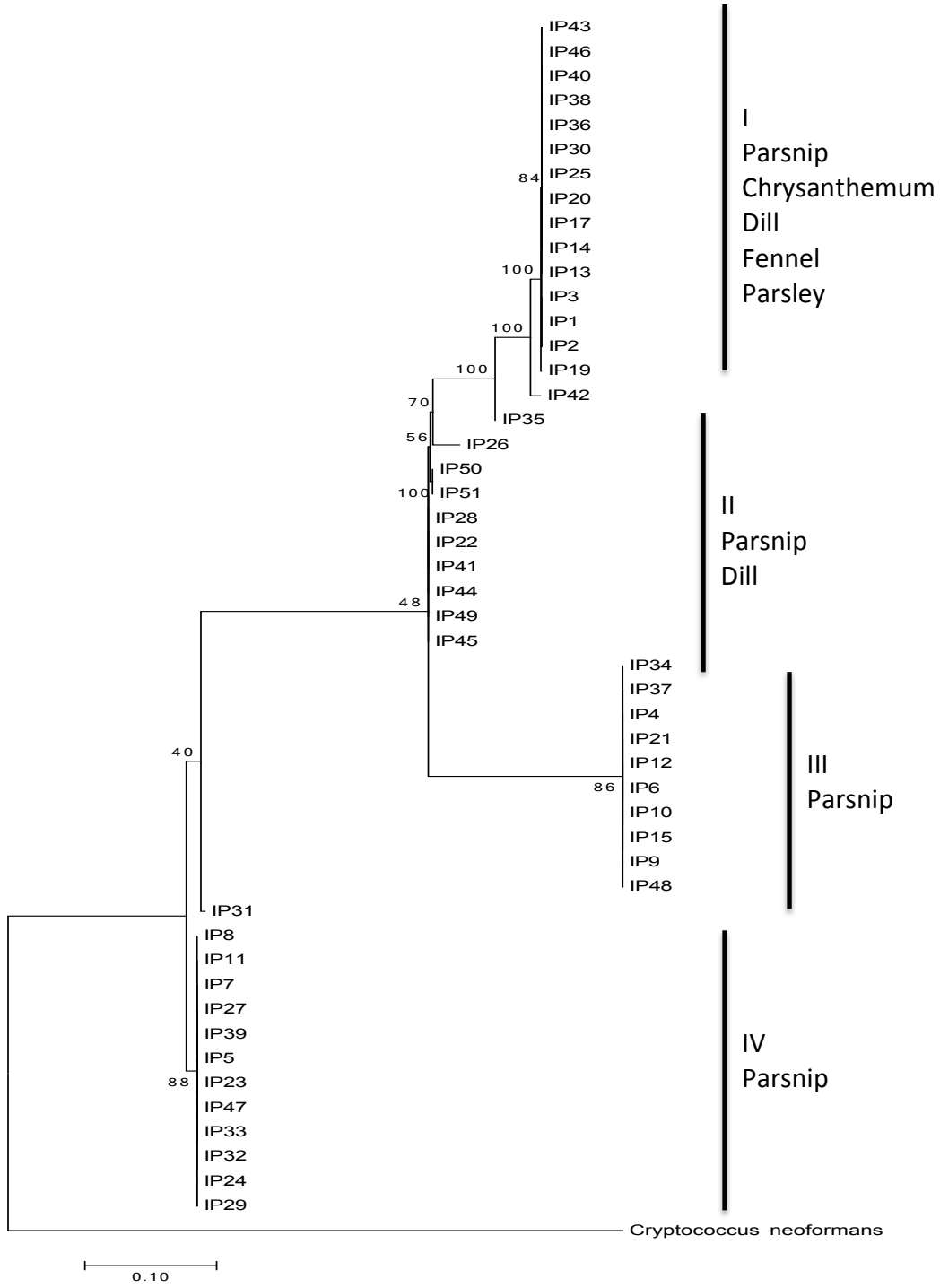


Figure 3.10 – Maximum Likelihood phylogenetic tree for *Itersonilia* spp. isolates based on the internal transcribed spacer (*ITS*), RNA polymerase II (*Rpb-II*), translation elongation factor (*EF-1α*), large ribosomal subunit (*LSU*), small ribosomal subunit (*SSU*) and beta-tubulin (*TUB2*). Numbers represent bootstrap values from 1,000 values. Scale bar indicates 0.10 substitutions per site. The tree is rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005).

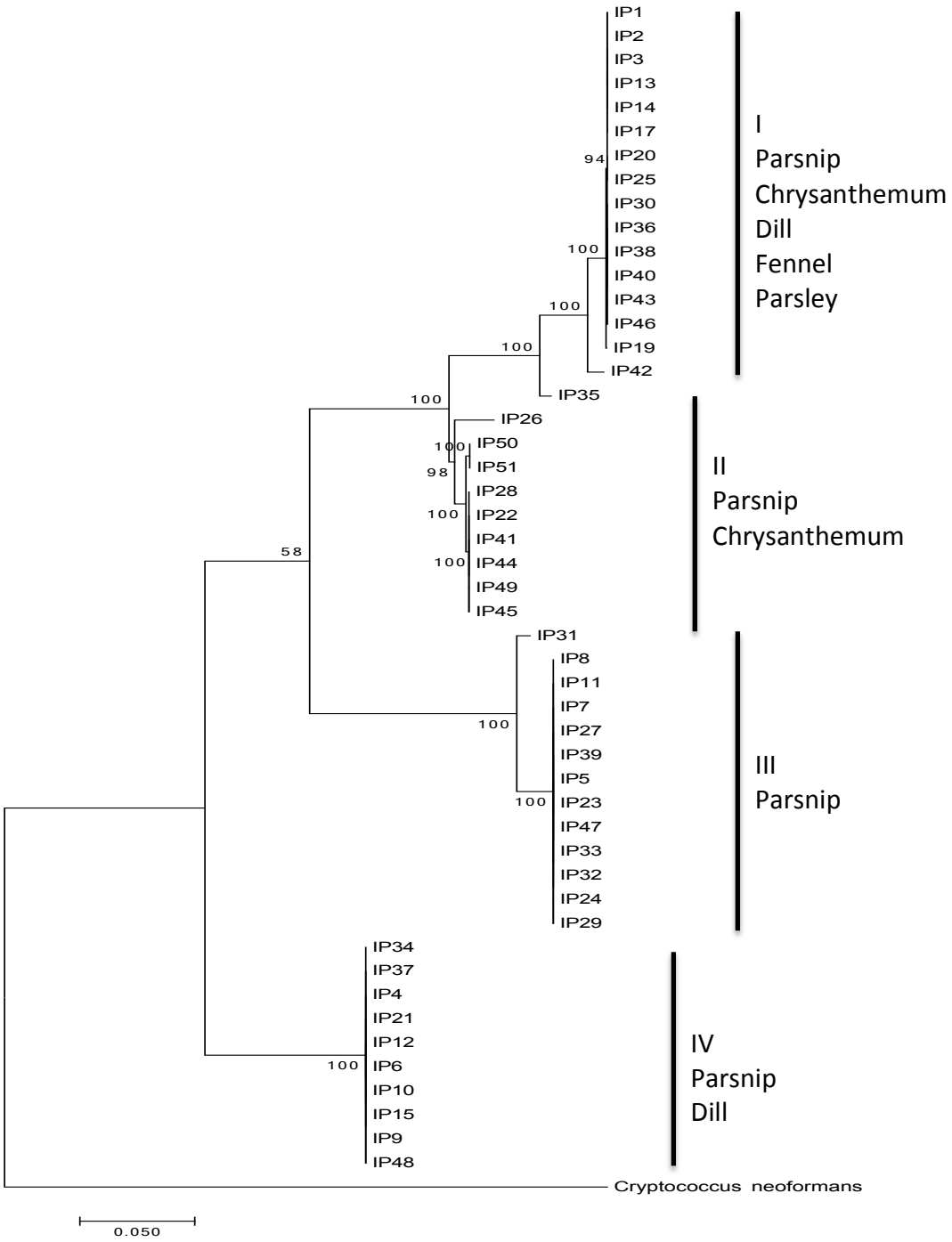


Figure 3.11 – Neighbour Joining phylogenetic tree for *Itersonilia* spp. isolates based on internal transcribed spacer (*ITS*), RNA polymerase II (*Rpb-II*), translation elongation factor (*EF-1α*), large ribosomal subunit (*LSU*), small ribosomal subunit (*SSU*) and beta-tubulin (*TUB2*). Numbers represent bootstrap values from 1,000 values. Scale bar indicates 0.050 substitutions per site. The tree is rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005).

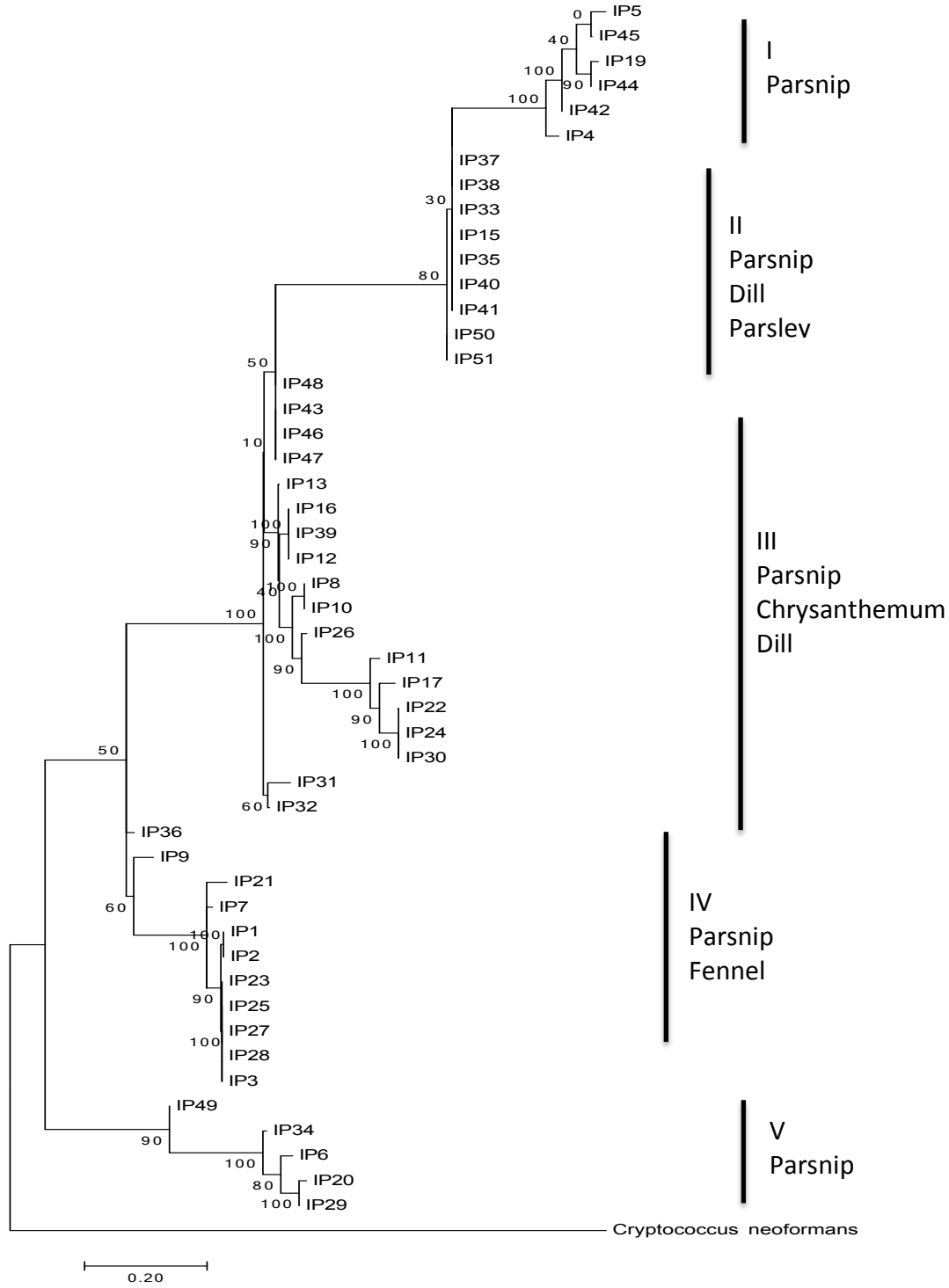


Figure 3.12 – Maximum Likelihood phylogenetic tree for *Itersonilia* spp. isolates based on the functional genes Triosephosphate transporter family (i), tRNA methyl transferase (*tMT*) and cellobiose dehydrogenase (*CDH*). Numbers represent bootstrap values from 1,000 values. Scale bar indicates 0.20 substitutions per site. The tree is rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005).

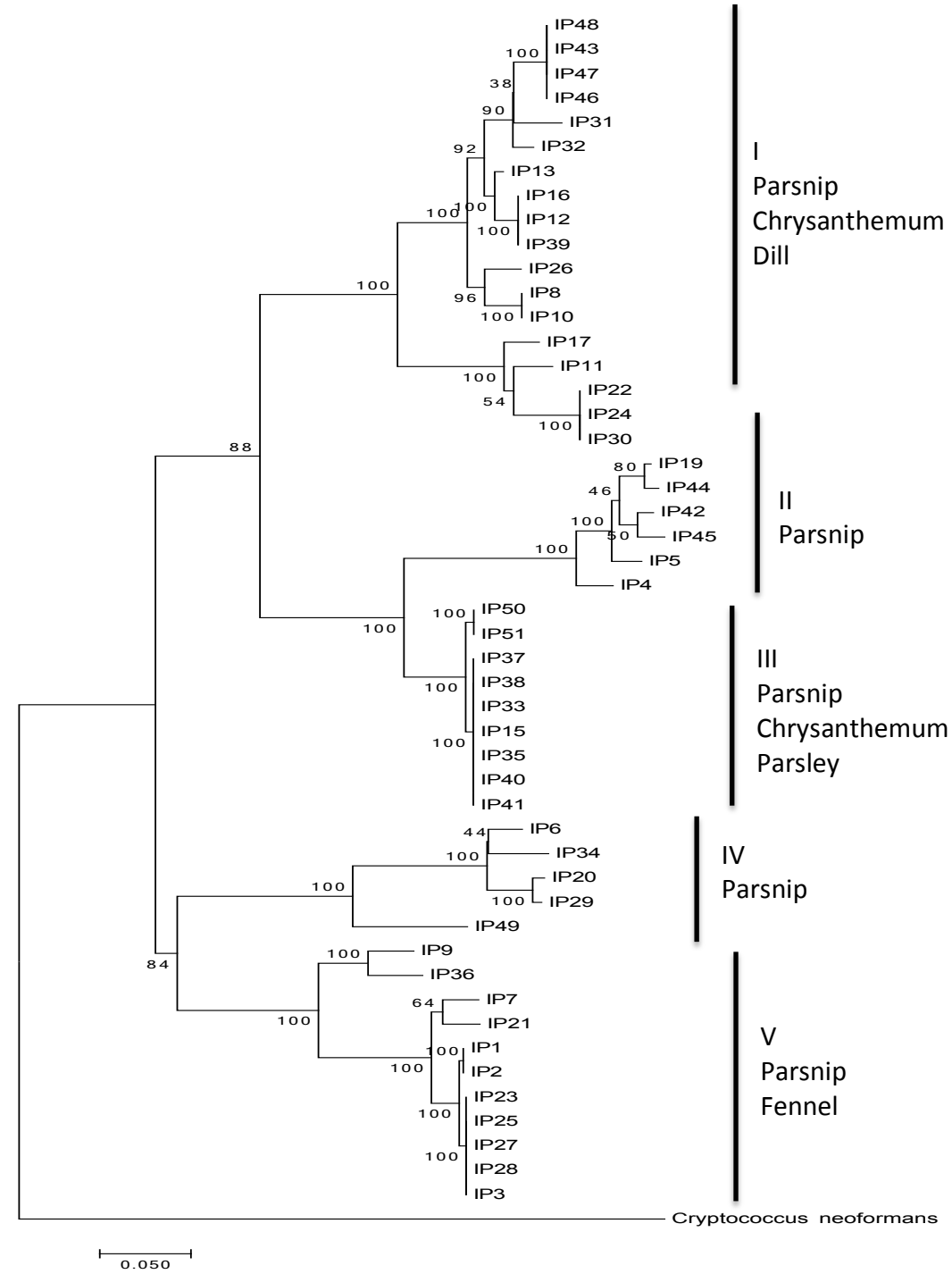


Figure 3.13 – Neighbour Joining phylogenetic tree for *Itersonilia* spp. isolates based on the functional genes Triosephosphate transporter family (*TTF*), tRNA methyl transferase (*tMT*) and cellobiose dehydrogenase (*CDH*). Numbers represent bootstrap values from 1,000 values. Scale bar indicates 0.050 substitutions per site. The tree is rooted through *Cryptococcus neoformans* (Loftus *et al.*, 2005).

3.3.6 Species-specific primer design for *Itersonilia* spp.

Primers designed from four functional genes were tested with DNA from a range of soilborne fungal pathogens to determine specificity. PCR amplification of primers 36F and 437R for the tRNA methyl transferase gene gave an amplicon size of 401 bp, and uniformly amplified all *I. pastinacae* isolates (amplified and sequenced for phylogenetic analysis, Section 3.2.7) acting as positive controls with no amplification in any of the other 57 fungal species tested (Table 3.4). Gel electrophoresis showed a clear band for *I. pastinacae* isolate IP10 (positive control) and no bands for any of the other fungi (Fig 3.14). PCR amplification of the tRNA methyl transferase gene was achieved with the thermocycling conditions: one cycle of 2 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 70°C and 1 min at 72°C followed by one cycle of 7 min at 72°C.

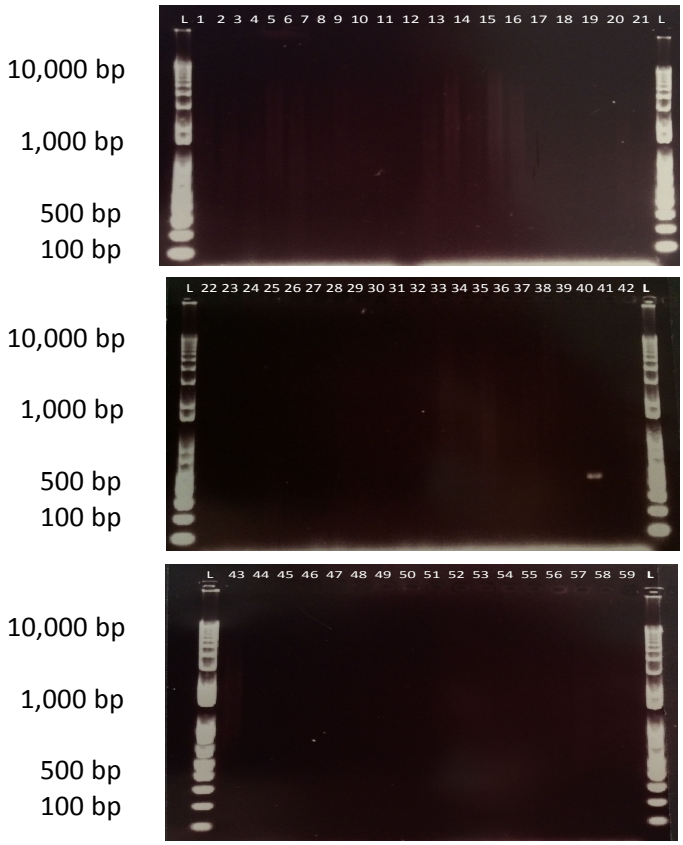


Figure 3.14 – Gel electrophoresis for PCR amplicons using *Itersonilia* spp. specific primers based on tRNA methyl transferase gene (*tMT*) for a range of fungal isolates (Table 3.4). Lane 1 – 58, fungal pathogens, Lane 40, *I. pastinacae* isolate IP10, Lane 59 negative control and Lane L, 1kb DNA ladder.

3.3.7 *I. pastinacae* gene expression analysis

Following transcriptome assembly of both the fungal and yeast phases of Itersonilia, gene expression analysis was carried out to identify the genes being comparatively up-regulated and down-regulated in each phase. This analysis represent a preliminary descriptive result with further work necessary to fully understand the gene regulation within *I. pastinacae*.

A total of 61 differentially expressed genes were identified from the *I. pastinacae* transcriptome analysis; 17 up-regulated in the fungal form (down-regulated in the yeast form) and 44 up-regulated in the yeast form (down-regulated in the fungal form) (Table 3.5). Of those up-regulated in the fungal form, two returned no result from the blastx analysis, five were identified as hypothetical proteins from a range of organisms the remaining 10 genes returned a positive blastx analysis with functions being annotated (Table 3.7). Two of the fungal up-regulated genes identified, g9431 and g9513, associated with lanosterol conversion and ergosterol biosynthesis respectively, code for enzymes associated with cytochrome P450. Whilst two of the other genes identified, g16384 and g5246 have functions associated with pectinesterase and nitric oxide dioxygenase.

Of the genes up-regulated in the yeast form (down-regulated in the fungal form), 14 returned no result from the blastx analysis and 14 were classified as hypothetical or unnamed proteins. The remaining 16 genes returned positive results from the blastx analysis with functions being annotated (Table 3.7). Of the positively annotated genes three are ABC transporter family genes (g15465, g8898 and g8956), two are transporter proteins (g14895 and g10211), one is a stress protein (g12682), one is a heat-labile enterotoxin (g7991) and g13585 is linked to melanin biosynthesis.

Table 3.7 – Differentially expressed genes in fungal and yeast forms of I. pastinacae, isolate IP10 detected using DESeq2. A total of 61 differentially expressed genes, 17 up-regulated in the fungal form and 44 up-regulated in the yeast form, with blastx annotation of genes.

Number	Gene	Normalised count (baseMean)	Fold change (log_2)	Standard error of fold change (log_2)	Wald statistic	p-value	p-adjusted value	Annotated Function	Accession number/ Best hit organism	E value
Fungal up-regulated genes										
1	g9431	1499.51	6.000	2.119	2.832	0.00463	0.0981	lanosterol 14 alpha-demethylase; Lanosterol demethylase	AKE49346.1: Xanthophyllomyces dendrorhou, CED84182.1:Xanthophyllomyces dendrorhous	3.00E-91
2	g6511	4534.43	6.034	2.104	2.868	0.00413	0.0981	hypothetical protein	KDQ18293.1: Botryobasidium botryosum FD-172 SS1	2.00E-23
3	g11179	955.08	6.227	2.162	2.880	0.00397	0.0981	laccase-1	XP_003711208.1: Magnaporthe oryzae 70-15, EHA51401.1: Magnaporthe oryzae 70-15	0.072
4	g9513	798.52	6.231	2.172	2.869	0.00411	0.0981	C22-sterol desaturase	AFV30193.1: Xanthophyllomyces dendrorhous, AFV30194.1: Xanthophyllomyces dendrorhous	2.00E-153
5	g16384	5669.96	6.316	2.130	2.965	0.00303	0.0931	pectinesterase-like; hypothetical protein	XP_004149498.2: Cucumis sativus, KGN49779.1: Cucumis sativus	6.7
6	g13267	11268.26	6.351	2.129	2.983	0.00286	0.0916	hypothetical protein	XP_016208623.1: Verruconis gallopava	3.90E+00
7	g5246	905.79	6.415	2.187	2.933	0.00336	0.0981	polysaccharide lyase family 1 protein	KIO16047.1: Tulasnella calospora MUT 4182	2.00E-23
8	g11276	1015.77	6.444	2.185	2.949	0.00318	0.0956	nitric oxide dioxygenase, Flavohemoprotein	WP_066226507.1: Bacillus sporothermodurans, KYD11255.1	5.00E-21
9	g6092	647.59	6.446	2.214	2.912	0.00359	0.0981	No Result	No Result	-
10	g15464	1860.11	6.529	2.171	3.007	0.00263	0.0916	hypothetical protein	ODV88036.1: Candida arabinofermentans NRRL YB-2248	7.9
11	g17736	10710.39	6.674	2.161	3.089	0.00201	0.0873	Armadillo-type fold	CED83792.1: Xanthophyllomyces dendrorhous	3.00E-19
12	g5161	2998.74	6.768	2.184	3.098	0.00195	0.0873	carbohydrate-binding module family 1 protein	KIO31155.1: Tulasnella calospora MUT 4182	8.00E-06

Number	Gene	Normalised count (baseMean)	Fold change (log_2)	Standard error of fold change (log_2)	Wald statistic	p-value	p-adjusted value	Annotated Function	Accession number/ Best hit organism	E value
13	g10983	753.62	7.481	2.347	3.187	0.00144	0.0792	hypothetical protein	KJA18950.1: Hypholoma sublateritium FD-334 SS-4	1.00E-11
14	g18791	780.79	7.532	2.351	3.204	0.00136	0.0792	hypothetical protein	ODO02849.1: Cryptococcus amylolentus CBS 6273	2.00E-39
15	g14515	591.67	7.547	2.391	3.157	0.00159	0.0792	general substrate transporter	KZV61327.1: Peniophora sp. CONT Length=516	2.00E-28
16	g10579	760.91	7.912	2.417	3.274	0.00106	0.0792	dehydrogenase	KZV68033.1: Peniophora sp. CONT Length=347	4.00E-05
17	g15303	2299.70	8.286	2.360	3.511	0.00045	0.0769	No Result	No Result	-
	Yeast up-regulated genes									
1	g14985	2087.08	-9.393	2.421	-3.880	0.00010	0.0769	No Result	No Result	-
2	g12790	819.98	-8.745	2.399	-3.645	0.00027	0.0769	metal ion transporter metal ion transporter	XP_007591711.1: Colletotrichum fioriniae PJ7	1.00E-09
3	g1775	1364.93	-8.573	2.356	-3.639	0.00027	0.0769	hypothetical protein	XP_003029455.1: Schizophyllum commune H4-8	5.00E-05
4	g15301	5688.26	-8.111	2.289	-3.544	0.00039	0.0769	unnamed protein product	CAK40084.1: Aspergillus niger Length=463	8.1
5	g221	3488.77	-7.949	2.279	-3.488	0.00049	0.0769	No Result	No Result	-
6	g10482	35400.64	-7.926	2.267	-3.496	0.00047	0.0769	No Result	No Result	-
7	g10252	2488.49	-7.789	2.268	-3.434	0.00060	0.0769	protein kinase C substrate, putative	XP_002478630.1: Talaromyces stipitatus ATCC 10500	3.5
8	g12682	1123.27	-7.736	2.280	-3.393	0.00069	0.0769	ribosomal protein L25/general stress protein Ctc	WP_019519059.1 50S: Moraxella boevrei	4.5
9	g17166	1011.62	-7.722	2.282	-3.384	0.00071	0.0769	No Result	No Result	-
10	g15178	2059.15	-7.712	2.264	-3.406	0.00066	0.0769	No Result	No Result	-
11	g7059	2571.49	-7.690	2.259	-3.404	0.00066	0.0769	hypothetical protein	KII86617.1: Plicaturopsis crispa FD-325 SS-3	2.00E-58
12	g16157	1481.68	-7.506	2.251	-3.335	0.00085	0.0792	No Result	No Result	-

Number	Gene	Normalised count (baseMean)	Fold change (log_2)	Standard error of fold change (log_2)	Wald statistic	p-value	p-adjusted value	Annotated Function	Accession number/ Best hit organism	E value
13	g6406	1868.41	-7.404	2.237	-3.310	0.00093	0.0792	hypothetical protein	OCF60652.1: Kwoniella mangroviensis CBS 10435	5.00E-20
14	g5800	972.72	-7.401	2.251	-3.288	0.00101	0.0792	No Result	No Result	-
15	g10653	6273.78	-7.335	2.220	-3.305	0.00095	0.0792	hypothetical protein	KNF01811.1: Puccinia striiformis f. sp. tritici PST-78	3.9
16	g14377	1696.78	-7.086	2.208	-3.209	0.00133	0.0792	hypothetical protein RSOLAG22IIIB_04136	CUA69880.1: Rhizoctonia solani Length=193	0.31
17	g11243	964.71	-7.065	2.218	-3.185	0.00145	0.0792	No Result	No Result	-
18	g18806	867.51	-7.064	2.221	-3.181	0.00147	0.0792	No Result	No Result	-
19	g2516	1621.51	-6.993	2.200	-3.178	0.00148	0.0792	hypothetical protein	OBR87228.1: Kwoniella dejecticola CBS 10117 Length=175	9.00E-22
20	g10211	3882.21	-6.957	2.188	-3.180	0.00147	0.0792	Permease of the major facilitator superfamily	CDZ97793.1: Xanthophyllomyces dendrorhous	5.00E-73
21	g16641	6163.37	-6.878	2.178	-3.158	0.00159	0.0792	No Result	No Result	-
22	g8897	620.08	-6.809	2.206	-3.086	0.00203	0.0873	RHTO0S04e01970g1_1	CDR39131.1: Rhodotorula toruloides Length=1481	1.00E-07
23	g15465	1447.31	-6.721	2.176	-3.089	0.00201	0.0873	ABC transporter G family member 11	CCO35884.1: Rhizoctonia solani AG-1 IB	7.00E-37
24	g17693	1380.18	-6.652	2.170	-3.066	0.00217	0.0904	hypothetical protein	KDE06938.1: Microbotryum lychnidis-dioicae p1A1 Lamole	2.00E-23
25	g14508	1519.63	-6.493	2.153	-3.016	0.00256	0.0916	hypothetical protein	EUC57310.1: Rhizoctonia solani AG-3 Rhs1AP, KEP45646.1: Rhizoctonia solani 123E	0.61
26	g5311	2650.29	-6.482	2.145	-3.021	0.00252	0.0916	No Result	No Result	-
27	g8898	1182.77	-6.453	2.153	-2.997	0.00272	0.0916	ABC transporter	XP_016271754.1: Rhodotorula toruloides NP11, EMS20635.1: Rhodotorula toruloides NP11	3.00E-34
28	g17129	2090.80	-6.453	2.145	-3.009	0.00263	0.0916	calcium-transporting atpase 3	CDZ97841.1: Xanthophyllomyces dendrorhous Length=1091	5.00E-35

Number	Gene	Normalised count (baseMean)	Fold change (log_2)	Standard error of fold change (log_2)	Wald statistic	p-value	p-adjusted value	Annotated Function	Accession number/ Best hit organism	E value
29	g8957	4355.42	-6.426	2.137	-3.007	0.00264	0.0916	ABC transporter G family member 11	CUA70558.1: Rhizoctonia solani Length=1456	2.00E-77
30	g1507	2213.56	-6.409	2.140	-2.995	0.00275	0.0916	No Result	No Result	-
31	g13833	1198.17	-6.396	2.147	-2.979	0.00289	0.0916	polysaccharide lyase family 1 protein	KIY68468.1: Cylindrobasidium torrendii FP15055 ss-10	2.00E-04
32	g11205	2330.26	-6.355	2.134	-2.978	0.00291	0.0916	hypothetical protein	OCF34273.1: Kwoniella heveanensis BCC8398 Length=900	6.1
33	g17715	840.81	-6.210	2.135	-2.908	0.00363	0.0981	hypothetical protein	KXS12158.1: Gonapodya prolifera JEL478 Length=1116	2.2
34	g2341	584.71	-6.196	2.145	-2.889	0.00386	0.0981	No Result	No Result	-
35	g11646	1056.82	-6.188	2.128	-2.907	0.00364	0.0981	glycoside hydrolase family 16 protein	XP_007771344.1: Coniophora puteana RWD-64-598 SS2	1.00E-27
36	g6789	2764.56	-6.178	2.116	-2.920	0.00350	0.0981	No Result	No Result	-
37	g987	2158.54	-6.124	2.112	-2.899	0.00374	0.0981	hypothetical protein	KDE05910.1: Microbotryum lychnidis-dioicae p1A1 Lamole	3.00E-25
38	g18442	4391.08	-6.039	2.099	-2.877	0.00402	0.0981	hypothetical protein	EPQ64895.1: Blumeria graminis f. sp. tritici 96224	5.1
39	g1181	708.18	-6.023	2.120	-2.840	0.00451	0.0981	WD40 repeat-like protein, partial	KWU41922.1: Rhodotorula sp. JG-1b Length=872	1.2
40	g1676	657.13	-6.014	2.122	-2.835	0.00459	0.0981	No Result	No Result	-
41	g7991	3427.75	-6.002	2.097	-2.862	0.00421	0.0981	Heat-labile enterotoxin, A chain	XP_011410633.1: Metarhizium robertsii ARSEF 23	5.3
42	g13052	4808.98	-5.946	2.090	-2.845	0.00443	0.0981	bmr1-like protein	XP_007284882.1:Colletotrichum gloeosporioides Nara gc5, ELA26070.1: Colletotrichum gloeosporioides	3.00E-34
43	g12349	2044.51	-5.934	2.094	-2.834	0.00459	0.0981	hypothetical protein	CED82661.1: Xanthophyllomyces dendrorhous Length=84	3.00E-04
44	g13585	3313.57	-5.919	2.089	-2.834	0.00460	0.0981	hypothetical protein	CEL63115.1: Rhizoctonia solani AG-1 IB	4.00E-10

3.4 Discussion

To date, there have been very few studies concerning the diversity and characteristics of *Itersonilia* species, with no specific research for isolates causing disease on parsnip since the work of Channon (1956, 1963a, 1981). Defining the biological characteristics and variability of *I. pastinacae* is important in understanding its epidemiology, assessing disease risk and developing control methods. The research done here on *I. pastinacae* may also be applicable to *Itersonilia* spp. on other crops.

In the root pathogenicity assay, all Itersonilia isolates, including those not isolated from parsnip, led to the development of lesions on parsnip roots. No significant differences were detected between the isolates, and isolates from other hosts were evenly distributed through the dataset, indicating they are all equally pathogenic on parsnip roots. In the leaf pathogenicity assay, Itersonilia isolates from all hosts led to the development of nectrotic lesions on parsnip leaves. Unlike for roots, in the leaf pathogenicity assay significant differences were detected, suggesting some isolates may be more pathogenic on parsnip foliage than roots. The current hypothesis on the transmission of *Itersonilia* spp. suggests that necrotic tissue from leaf lesions fall onto the soil surface and onto exposed shoulders of parsnip roots leading to the development of parsnip canker (Channon, 1963a; Gladders, 1997). Given this, *Itersonilia* spp. isolates that are more pathogenic on leaves will lead to larger necrotic lesions developing, with potentially more spores. When diseased tissue falls into the soil this would result in a higher inoculum load, potentially causing a higher number of root lesions. This means Itersonilia isolates that are more pathogenic on leaves could be more pathogenic on roots through increased inoculum load in the soil.

Within the leaf pathogenicity assay, isolates from hosts other than parsnip range from weakly pathogenic to strongly pathogenic, suggesting there is no specialism based on host, although it should be noted the two most pathogenic isolates in

this assay were from parsnip. Based on the work of Channon (1963a) and Sowell & Korf (1960), who investigated host specificity of Itersonilia, it could be hypothesised that Itersonilia from parsnip hosts would be more aggressive on parsnip roots and leaves. However, from these two pathogenicity assays there is no evidence of isolates from parsnip being more aggressive than isolates from any other host.

Experiments assessing the effect of temperature on the growth rate of *Itersonilia* spp. isolates indicated that the lowest growth rate across all isolates was at 0°C and the highest growth rate between 15 and 25°C. For the standard isolate IP10, the lowest rates of growth were at 0 °C and 30 °C, whilst the maximum growth rate was at 20°C. This is the first comprehensive study on the effect of temperature on growth of *Itersonilia* spp. Previous work by Ingold (1984) found that the growth rate of Itersonilia on malt agar at 20°C had a radial extension of about 2 mm day^{-1}. However, Ingold (1984) only measured one isolate, and a more comprehensive study was needed. In fact, the research conducted here found the average rate of growth across all isolates to be 3.1 mm day^{-1}, with individual isolate growth rates ranging from 1.82 to 4.41 mm day^{-1}, thus locating the values measured by Ingold (1984) in the low end of the growth rate spectrum.

The parameter estimates from the model fitting on observed growth rates varied significantly between the model fitted to all *Itersonilia* spp. isolates and the standard isolate IP10. It is not appropriate to fit this model to all isolates simultaneously because the high viability between isolates lead to biologically unrealistic estimates. However, fitting the model to one isolate only gives more biologically realistic estimates, particularly when a wide range of temperatures is investigated.

It is generally accepted that the majority of saprophytic fungi do not grow below 5°C (Ayrest, 1969). However, all Itersonilia isolates were found to grow at 0°C.

This result is in line with the taxonomy of Itersonilia within the Cystofilobasidiales order, a group of cold yeasts, including *Mrakiella cryoconiti* and *Mrakia frigida*, which are able to maintain growth at temperatures as low as -12°C (Margesin & Fell, 2008; Ming-Xiu & Pei-Jin, 2007). The incidence of Itersonilia and parsnip canker has also previously been found to increase in colder conditions, whilst the cut flower industry has found inflorescence symptoms to increase in number and severity post harvest, when refrigerated at temperatures of 1°C (Channon, 1963a; Gandy, 1966). Parsnips are a predominantly winter crop and, with average winter temperature ranges between -1°C and 6°C across the UK, the ability of *Itersonilia* spp. to maintain growth at low temperatures such as these explains the prevalence of cankers in parsnip crops during prolonged periods of cold. In addition, as mentioned above, few fungi can grow at temperatures below 5°C. It is therefore probable that the increase in *Itersonilia* spp. incidence over winter may also be due, in part, to reduced activity and competition from other saprophytic fungi. It is important to note, however, that whilst Itersonilia does continue to grow at cold temperatures, the average rate of growth at 20°C is much lower than that of other parsnip pathogens such as *C. destructans* and *M. acerina*, which display an average rate of growth at 20°C of 5.68 mm day^{-1} and 5.57 mm day^{-1} respectively (Sections 4.3.3 and 4.3.8, Chapter 4).

In assessing the effect of temperature on spore production, chlamydospores were found to be more abundant than ballistospores at 0 to 5°C, whilst ballistospore densities were higher than chlamydospores between 10 and 25°C. Ballistospores are known to be discharged from parsnip seed and foliage, and are generally considered a mode of asexual reproduction, whilst chlamydospores are found in the soil and are survival structures to maintain the pathogen from one crop to the next (Channon, 1963a; Sowel & Korf, 1960). It could therefore be hypothesised that ballistospores are more abundant at higher temperatures as the primary infection propagule of parsnip foliage and seed during the summer, whilst chlamydospores would be more abundant at lower temperatures as the

primary infection propagule of parsnip roots during the winter. The research conducted here confirms this hypothesis, as chlamydospore numbers were greater than ballistospores at low temperatures and ballistospore numbers greater than chlamydospores at higher temperatures. However, the density of ballistospores was observed to remain relatively constant over the six temperatures investigated, rather than reducing at lower temperatures, suggesting that the fact that *Itersonilia* spp. have been detected on cut flowers at 1°C, and on plant debris throughout winter (Gandy, 1966) is more likely due to an increase in chlamydospores production as opposed to a reduction in ballistospore production.

The understanding of which spore types are more prevalent at certain temperatures is also important commercially. As ballistospores are seed-borne, they cycle around parsnip foliage and seed, and cause infection in seedlings (Smith, 1966). This poses a problem for both domestic and commercial seed producers. For growers producing their own seed this is particularly problematic as a biennial crop seed set occurs in the second year. If roots develop Itersonilia infection during the winter, this could potentially lead to increased inoculum the following spring when seed sets, resulting in seed coated in an increased Itersonilia inoculum. For commercial seed producers it is important to provide growers with clean seed. As shown by Smith (1966), steam treatment of 45°C for 30 minutes eliminates *Itersonilia* spp. from the seed coat, resulting in reduced seedling infection. Commercial seed producers treat, and frequently coat, seed to eliminate Itersonilia inoculum, and seedling infection.

The knowledge of spore relative prevalence at certain temperatures could also be used to more accurately apply fungicides and control methods, such as crop rotations, to reduce the spread of inoculum. As ballistospores have been identified in all *Itersonilia* spp., and have been found in isolates from a range of hosts, including weed species (Gandy, 1966), field margins could be a possible source of inoculum, in addition to parsnip foliage and seed. By controlling

ballistospore release, the levels of Itersonilia inoculum could be significantly reduced. It is clear they are present at temperatures from 0°C to 25°C and, through spore trapping, have been identified as more prevalent in mornings with heavy dew, and following irrigation (Channon, 1963b; Gandy, 1966). By applying fungicide early in the morning or following irrigation, it may be possible to reduce infection through the spread of ballistospores. In addition, as ballistospores were identified across a range of temperatures, it is clear there is no specific time of the year when they are not being produced, although the lack of foliage in winter would limit their spread. This would perhaps support a regular fungicide programme, based on temperature and relative humidity, to control levels of inoculum. Given the importance of ballistospores in the transmission of Itersonilia, there is a need to rapidly detect the presence of the pathogen. The PCR-based diagnostic assay developed here will allow rapid diagnostics for the presence of Itersonilia in plant material, soil or on the seed. This is particularly relevant for commercial seed companies, where clean, pathogen-free seed must be reliably delivered to growers. Conventional seed testing takes five days, and can be unreliable if both the parsnip seed and fungal colonies are not examined thoroughly. The use of Itersonilia specific primers in a PCR diagnostic assay would increase productivity and accuracy for commercial seed companies. As seen in the trapping and quantification of *Sclerotinina sclerotiorum* ascospores in *Brassica napus* crops (Rogers *et al.*, 2009), this method could be further developed into a quantitative PCR (q-PCR) and used alongside spore trapping and soil sampling to quantify the levels of ballistospores within the foliar canopy and number of chlamydospores within the soil, to further advise control methods and fungicide applications.

As previously mentioned, the root and leaf pathogenicity assays identified all *Itersonilia* spp. isolates from all hosts to be capable of infecting both parsnip roots and leaves, resulting in the development of necrotic lesions. One of the characteristics factors in discriminating between Itersonilia species is that an isolates obtained from one host is non-pathogenic to other host species; for

examples an isolate from parsnip is non-pathogenic to chrysanthemum, and vice versa (Channon, 1963b; Sowell & Korf, 1960). This is in direct contrast to the results of this study, where isolates from other hosts, including chrysanthemum, dill, parsley and fennel, were shown for the first time to be able to infect both parsnip roots and leaves. As a direct contradiction to one of the key discriminating factors between Itersonilia species, it brings into question the concept of two separate species. Further to this, the results from the experiments into the effect of temperature on spore production found all *Itersonilia* spp. isolates to produce both chlamydospores and ballistospores, with no difference in spore numbers related to host. According to Channon (1956), the other key factor in discriminating between *Itersonilia* spp. isolates is the abundant production of chlamydospores in *I. pastinacae* isolates from parsnip whilst isolates from other hosts rarely producing any chlamydospores. The results in this study are again a direct contradiction to the work of Channon (1956), further questioning the concept of two separate species. Finally, the phylogenetic analyses of the six housekeeping and three housekeeping genes identified diversity between the *Itersonilia* spp. isolates. Each clade contained isolates from a range of locations, and isolates from the same location featured in different clades: for example, three Itersonilia isolates from dill, fennel and parsley (IP36, IP37 and IP38) from a farm in Middlesex are not found together in a single clade. It might be expected that this was a single introduction of Itersonilia that spread across all available hosts, however even in the *ITS* phylogeny only two of these isolates (IP36 and IP37) are grouped together. This indicates that the distinction between isolates was not based on geographical location. Within the six loci housekeeping gene phylogenies, two clades from the maximum likelihood analysis and one clade from the neighbour joining analysis are comprised of isolates from only parsnip hosts. In the three loci functional gene analysis two different clades in each analysis (maximum likelihood and neighbour joining) are comprised solely of isolates from parsnip hosts. All other clades comprise isolates from a variety of other hosts (chrysanthemum, dill, fennel and parsley) suggesting the distinction between isolates is not based on

host. Based on all these data, there is little evidence for these isolates to be classed as two separate species. However, to fully determine the phylogenetic analyses, further isolates from hosts other than parsnip would help to understand if those clades comprising isolates only from parsnip hosts are fully resolved. In addition, further assays to determine the pathogenicity of Itersonilia isolates from parsnip hosts on other host species would be required to confirm the separation of Itersonilia into two different species is unsubstantiated.

The gene expression analysis of the Itersonilia fungal and yeast phase highlighted a total of 61 differentially expressed genes; 17 of which were up-regulated in the fungal phase (down-regulated in the yeast phase) and 44 up-regulated in the yeast phase (down-regulated in the fungal phase). Two of the genes up-regulated in the fungal phase (g9431 and g9513) encode a cytochrome P450 enzyme associated with ergosterol biosynthesis. The best hit organisms associated with this annotated function is *Xanthophyllomyces dendrorhou*, a basidiomycetous yeast. The protein is highly conserved, and thought to be involved in the last step of the ergosteol biosynthesis conversion pathway (Loto *et al.*, 2012). In Xanthophyllomyces this protein appears to be closely linked to carotenoid production. However, in higher fungi, it is thought to be related to climatic instabilities (Dupont *et al.*, 2012). As *Itersonilia* spp. is from the order Cystofilobasidiales, a group of arctic yeast (Millanes *et al.*, 2011), and as shown in the experiments here assessing the effect of temperature on growth rate, Itersonilia is able to maintain growth at low temperatures. Therefore, the up-regulation of this gene in the Itersonilia fungal phase might be associated with changes in climate, such as relative humidity.

Two other genes of particular interest that have ben identified as up-regulated in the fungal phase are g16384 and g5246: these code for pectinase-like and polysaccharide lyase family proteins, respectively. Pectinase and polysaccharide lyase proteins have been found in plant pathogens such as *Fusarium graminearum* and *Phytophthora infestans* to play a role in polysaccharide

degradation for cell wall disruption in plants (Zhao *et al.*, 2013; Förster & Mendgen, 1987). These two genes do not appear to be up-regulated in the fungal phase of Itersonilia and, as shown by Boekhout (1991), Itersonilia in the yeast phase is non-pathogenic on parsnip. It could therefore be hypothesized that these two genes coding for pectinase and lyase family proteins could be involved in the pathogenicity of Itersonilia in the fungal form on plant material, given they are up-regulated in the fungal phase, and not up-regulated in the yeast phase.

Three of the 44 genes identified as up-regulated in the yeast phase code for ABC transporter G family members. These genes play an essential role in membrane transport, and are well characterised in vertebrates to play an essential role in lipid transport and metabolism. The only research carried out on the function of this sub-family in relation to fungi is in *Magnaporthe grisea*, a rice pathogen, where they are required for pathogenesis and development of appressoria (Kovalchuk *et al.*, 2013), although they have been found in other plant pathogens too (Perlin *et al.*, 2014). However, as no research to date has confirmed the pathogenicity of Itersonilia in the yeast phase, the specific function of these genes in Itersonilia cannot be confirmed.

Gene g10252 is up-regulated in the yeast form and associated with protein kinase C gene. Protein kinase C is known to play an important role in the control of proliferation and differentiation of a wide range of cell types, with suggestions this enzyme is involved in the control of morphogenetic transitions in dimorphic fungi (Piñero & Vallo, 1997). However, further analysis would be required to support the possible involvement of this important signal transduction enzyme in the control of morphogeneis in *Itersonilia* spp.

In conclusion, this work required the development of novel robust methods to assess the pathogenicity of *Itersonilia* spp. isolates on parsnip roots and seedlings, and represents the first analysis where isolates from other hosts have

been found to cause canker-like symptoms on parsnip. It is also the first time the effect of temperature on growth rate and spore production has been quantified. The results confirm *Itersonilia* spp. are able to grow at low temperatures, with isolates from all hosts producing higher numbers of chlamydospores at low temperatures and higher numbers of ballistospores at high temperatures. Both of these aspects are consistent with the hypothesised mode of transmission, and clearly explain the prevalence of parsnip canker caused by Itersonilia during the winter season.

This study is also the first one where transcriptome sequencing and gene expression analysis of the fungal and yeast phase of *Itersonilia* spp. were performed. Up-regulated and down-regulated genes of interest were identified, possibly relating to pathogenicity and the morphogenic transition from the fungal to yeast phase. In addition, the specific PCR assay developed here has commercial potential for rapid and reliable detection of *Itersonilia* spp. from plant tissue, seed and soil to improve the productivity and accuracy in commercial seed testing. The results of this novel analysis provide a more comprehensive understanding of the biological and molecular characteristics of *Itersonilia* spp. Although more work needs to be done in assessing the pathogenicity of parsnip-derived *Itersonilia* spp. isolates on other hosts, the results of the pathogenicity assays and molecular analysis conducted here do not support the current distinction between Itersonilia species, suggesting the description of Itersonilia as a single species may be more appropriate.

4. Characterisation of *Cylindrocarpon destructans* and *Mycocentrospora acerina* isolates from parsnip

4.1 Introduction

C. destructans and *M. acerina* are known plant pathogens of parsnip, causing orange and black root cankers respectively, which can lead to significant crop losses (Channon, 1965, 1981; McPherson, 2013). Little research has been carried out into these two fungal pathogens, and no research to date has investigated the characteristics and diversity of *C. destructans* and *M. acerina* isolates derived from parsnip hosts.

C. destructans is part of the Nectriaceae family of the Hypocreales order, a group of fungi containing a number of plant pathogens including *Cylindrocarpon* species, which are responsible for infecting a range of trees and woody plants (Castlebury *et al.*, 2006), such as beech, grapevine, pine, ginseng and apple causing symptoms such as bark canker, black foot rot and root disease respectively (Castlebury *et al.*, 2006; Alaniz *et al.*, 2007; James, 2008; Braun, 1995). Historically, such brightly coloured and uniloculate, perithecial ascomycetes were classified in the genus Nectria of the Hypocreales order, where approximately 125 *Cylindrocarpon* spp. have been described to date (Mantiri *et al.*, 2001). However, recent taxonomic studies have attempted to redefine Nectria, with many species being redistributed among genera in both the Bionectriaceae and Nectriaceae (Rossman *et al.*, 1999). In addition, many teleomorphs and anamorphs exist within the Nectria group (Samuels *et al.*, 2009). *Neonectria radicicola* and *Ilyonectria radicicola* represent that same species and are teleomorphs, whilst *C. destructans* is described as the anamorph

of this species, as previously mentioned in Chapter 1 (Section 1.4.1, Table 1.2). This leads to further confusion when confirming species identity.

Cylindrocarpon species are generally considered weak or minor pathogens with limited importance on vegetables, small fruits, fruit trees, forage plants, ornamentals and conifers (Mantiri *et al.*, 2001). Of these sectors, grapevines are of the most economic importance (Oliveira *et al.*, 2004) and therefore little research has been carried out in relation to other host plants.

Isolates of *Cylindrocarpon* have traditionally been identified by type of symptoms on plant hosts, colony morphology and reproductive characters following isolation (Fox & Narra, 2006). This is, however, prone to inaccuracy due to the occurrence of fast growing saprophytes also present within diseased tissue, and changes in symptomology due to environmental factors such as extreme temperatures (Probst, 2011). Moreover, colony morphology is often insufficient to differentiate species, and hence gene sequence data is now being used to supplement traditional morphological techniques (Petit & Gubler, 2005). The *ITS* (Internal transcribed spacer) regions of the rRNA genes have been used to identify *Cylindrocarpon* spp., but frequently fail to differentiate between closely related species due to insufficient polymorphism (Jones, 2001). In such cases, other DNA regions such as the Beta-tubulin (*TUB2*) and translation elongation factor (*EF-1*α) are also commonly used (Aroca & Raposo, 2007).

The mode of infection of parsnip roots by *C. destructans* is not fully understood, but a combination of favourable conditions including temperature, humidity and root exudates are likely to be responsible for the germination of chlamydospores and production of hyphae resulting in the invasion of plant material (Horsfall & Dimond, 1960). Whilst no research has been carried out to determine the pathogenicity of *C. destructans* isolates from parsnip, there is evidence that isolates within the same *Cylindrocarpon* species may differ in their pathogenicity. For instance, Seifert *et al.* (2003) reported significant differences between

virulence of *C. destructans* isolates in ginseng and conifer, as did Alaniz *et al.* (2009) for isolates of *C. macrodidymum* in grapevine. However, research by Petit & Gubler (2005) found no difference between *C. destructans* or *C. macrodidymum* in their ability to infect grapevine, with all isolates causing significant root rot symptoms. Probst (2011) noted that investigations into the relative pathogenicity of fungal isolates required the development of a reliable and reproducible infection method leading to the development of symptoms similar to those observed by growers during natural infection.

Channon (1981) isolated a total of 20 *C. destructans* isolates from black, brown shoulder cankers on parsnip roots. He then tested eight of these isolates for pathogenicity of parsnips, by inserting agar plugs into slits in the shoulders of the roots. Results varied in virulence, identifying four highly pathogenic and four weakly pathogenic isolates, with all symptoms identical to those on the original roots. This work proved *C. destructans* can cause canker symptoms on parsnip roots. However, it was not possible to ascertain from this study if *C. destructans* was a primary pathogen able to infect undamaged root tissue, as the roots these isolates were originally isolated from reported extensive damage from carrot root fly larvae (Channon, 1981).

Whilst no studies have been carried out into the relative pathogenicity of *C. destructans* isolates from parsnip since the work of Channon (1981), there have been studies characterizing *Cylindrocarpon* spp. associated with black foot disease of grapevines. Pathogenicity assays inoculating grapevine canes with 10 day old mycelial plugs led to vascular discoloration with different isolates causing different levels of pathogenicity (Úrbez-Torres *et al.*, 2014). Chlorotic leaves with scorching at the edge were also observed in a number of the plants, with a substantial reduction in vigour, and reduced shoot growth also documented (Úrbez-Torres *et al.,* 2014). Whilst grapevine root dipping experiments led to root lesion symptoms and vascular discoloration. Strains of *C. destructans* from different provinces were documented as showing a great variety in morphology

and pathogenicity on grapevine, meaning patterns of variation were difficult to identify, especially when only the anamorph characters were considered (Rego *et al.*, 2001). Alaniz *et al.* (2007) identified a range of virulence between *Cylindrocarpon* spp. isolates following root dipping, and it was also noted that *Cylindorcarpon macrodidymum* and *Cylindrocarpon liriodendri* spp. isolates from a range of geographical locations had similar beta-tubulin gene sequences and were grouped together in a single clade. Following experiments to determine the effect of temperature on radial colony growth at seven temperatures (5, 10, 15, 20, 25, 30 and 35°C), colony morphology was found to be highly heterogeneous, with isolates growing at all temperatures, except 35°C. Sporulation experiments by Alaniz *et al.* (2007) also found isolates to show a wide range of variation in total spore produced.

Mycocentrospora acerina is in the order Capnodiales, a group incorporating plant and human pathogens, endophytes, saprobes and epiphytes with a wide range of nutritional modes. In addition to the Mycosphaerellaceae the order also contains Teratosphaeriaceae and Schizothyriaceae, all families containing plant pathogens of major importance (Crous *et al.*, 2009). Cercospora, a genus of the Mycosphaerellaceae is one of the most important genera of pathogenic fungi in agriculture, with most members having a very broad host range across all major families of dicotyledons, most monocotyledons, some gymnosperms and ferns (Pollack, 1987). Cercospora species, including *M. acerina*, are commonly associated with leaf spots, but can also cause nectrotic lesions on flowers, fruit, bracts, seeds and pedicles of numerous hosts in most climatic regions (Agrios, 2005). In addition, several species of this genus are also known hyperparasites of other plant pathogenic fungi (Shin & Kim, 2001), and have been used as biocontrol agents of weeds (Morris & Crous, 1994).

The current host range of *M. acerina* consists of 90 plant species comprising common weeds and vegetable crops including carrot, celery, spinach and peas (Tompkins & Hansen, 1950). These alternative hosts are thought to play a vital

role in the survival of the fungus in the field due to the abundance of long-lived chlamydospores (as described in Chapter 1) produced in senescing tissue. It is common agricultural practice to plough in this infected material, hence leading to increased inoculum levels in soil (Day *et al.*, 1972; Wall & Lewis, 1978). While the mode of infection through which *M. acerina* infects parsnips is not fully understood, Wall & Lewis (1979) demonstrated a high correlation between levels of chlamydospore inoculum in the soil at time of sowing and subsequent infection in the carrot crop, while Newhall (1944) stated that a three-year rotation of celery was not sufficient to eliminate infection from *M. acerina* chlamydospores in the soil. Research on pathogen resistance demonstrated that the periderm of carrots was involved in resistance to a wide range of pathogens, including *M. acerina*. Infection in carrot was almost always associated with root damage and wounding, suggesting mechanical, as well as other pathogens or insect damage, could be partially responsible for *M. acerina* infections (Davies & Lewis, 1981). Therefore, it could be hypothesised that the increased conidia production during the spring and autumn (Arsvoll, 1969) results in aerial transmission throughout the summer when foliage is present, whilst the abundance of chlamydospores produced in senescing tissue throughout autumn (Day *et al.*, 1972; Davies, 1977) results in necrotic tissue falling into the soil leading to a build up of inoculum, in a similar mode of transmission to *Itersonilia* spp.

Channon (1965) first described *M. acerina* as a cause of parsnip canker, and described the morphological and pathogenic characteristics of the isolates to be similar to those obtained from carrots and celery in the UK. *M. acerina* isolates from parsnip, carrot and celery were used in pathogenicity experiments, where parsnip, carrot and celery were cross-infected in all combinations by placing inoculated beet seed against the shoulders and petiole base, respectively. The inoculated sites were then re-covered with compost, and assessed for root and petiole disease. Both parsnip and celery developed extensive cankers, whilst carrots developed less pronounced, dry, shallow lesions. Isolates from parsnip

and carrot were generally more virulent, with a detected difference in the pathogenicity of the two isolates from parsnip (Channon, 1965).

In assessing the effect of temperature on *M. acerina* growth rate, Channon (1965) cultured isolates from parsnip, carrot and celery hosts, at a range of temperatures between -0.7 and 29.0°C. All isolates displayed similar growth-temperature curves where maximum growth was achieved in the range 18.5-21.5°C. Research by Srivastava (1958) found *M. acerina* isolates from carrot led to the development of lesions in both wounded and unwounded carrots after four weeks at 18°C following inoculation with mycelium; in addition, the isolate was also found to be pathogenic on celery, where necrotic spots were produced within three days after spraying of a spore suspension onto 2 week old plants (Srivastava, 1958). In contrast, research by Iqbal & Webster (1969) assessed the pathogenicity of *M. acerina* isolates on carrot and parsnip, noting that mycelial inoculation led to lesions developing on both carrot and parsnip roots. It was noted the lesions on parsnip were more extensive, dark brown and soft, compared to the dry, shallow lesions on carrots (Iqbal & Webster, 1969).

Limited molecular data for understanding the taxomony of *M. acerina* exists, with only 70 *ITS* sequences for *M. acerina* available on GenBank. However, Stewart *et al.* (1999) examined the phylogenetic relationships of Mycosphaerellaceae, using common housekeeping genes, specifically rRNA and non-coding rDNA, to assess both conserved and variable regions for inferring phylogenetic relationships.

Other than the work of Channon (1965), little is understood about the characteristics and diversity of *C. destructans* and *M. acerina* isolates from parsnip roots. The aims of the work in this chapter are:

- To determine the pathogenicity of *C. destructans* and *M. acerina* isolates on parsnip roots and seedlings;

- To determine the effect of temperature on mycelial growth and spore production of different *C. destructans* and *M. acerina* isolates from parsnip;
- To investigate intra-species diversity within different *C. destructans* and *M. acerina* species, using housekeeping gene analyses.

4.2 Materials & Methods

4.2.1 Pathogenicity of *C. destructans* isolates on parsnip roots

Experiments were set up to assess the pathogenicity of *C. destructans* isolates on freshly harvested roots from commercial parsnip growers. A total of 16 Isolates from the 31 isolated from diseased parsnip roots (Chapter 1) were selected based upon geographic origin to reduce isolate duplication (Table 4.1). Parsnip roots (cv. Javelin) were mechanically harvested, washed with tap water, surface sterilized with 70% ethanol (v/v) and air-dried. Each root was checked for any disease symptoms before the widest part of the root was inoculated with a 5 mm agar plug of a *C. destructans* isolate from a seven-day-old actively growing colony, grown on potato dextrose agar (PDA) at 20°C, in dark conditions. Parsnips were then incubated at 12°C on damp tissue in sealed 3 L plastic boxes and moist conditions maintained to encourage disease development by misting roots with RO water once a week. Six replicate parsnip roots were set up for each *C. destructans* isolate, and three repeat experiments were carried out. Photographs of symptoms were taken weekly from three weeks post-inoculation and lesion area measured using ImageJ (https://imagej.nih.gov/ij/).

Table 4.1 – Isolate number and origin of *C. destructans* isolates characterised in this study.

C. destructans **isolate number**	**Isolation code**	**Site**	**Location**	**Country**
CD1	R1.2	1	Spalding, Lincolnshire	England
CD5	R3.10	1	Spalding, Lincolnshire	England
CD10	R8.6 (2)	1	Spalding, Lincolnshire	England
CD18	Cylindrocarpon 2	2	Spalding, Lincolnshire	England
CD19	R2 W10 B	2	Spalding, Lincolnshire	England
CD20	R13.2	3	Spalding, Lincolnshire	England
CD21	R22	3	Spalding, Lincolnshire	England
CD22	R1.1 (3)	4	Cupar, Fife	Scotland

CD23	R2.1	4	Cupar, Fife	Scotland
CD24	R3.1 (2)	4	Cupar, Fife	Scotland
CD25	R4.2	4	Cupar, Fife	Scotland
CD26	R7.2	4	Cupar, Fife	Scotland
CD27	R8.1	4	Cupar, Fife	Scotland
CD28	R9.2	4	Cupar, Fife	Scotland
CD30	Elsoms 2	5	Spalding, Lincolnshire	England
CD31	Elsoms 3	5	Spalding, Lincolnshire	England

4.2.2 Pathogenicity of *C. destructans* isolates on parsnip seedlings

Experiments were set up in the glasshouse to assess the pathogenicity of different *C. destructans* isolates on parsnip seedlings in controlled environment experiments using the same isolates as used in the parsnip root pathogenicity experiments (Section 4.2.1, Table 4.1). A total of 70 parsnip seedlings (cv. Panache), were grown from seed in a 2:1 mix of Levington F2 compost and sharp sand (Everris, UK) in 308 module trays placed in plastic bags to maintain humidity and increase soil temperature to encourage germination. Trays were placed in a glasshouse compartment at 20°C, and after three weeks plastic bags were removed from the trays. When seedlings were five weeks old they were then used in the pathogenicity tests. *C. destructans* isolates were subcultured onto PDA and grown for 10 days at 20°C. Five mycelial plugs from the leading edge were placed into sterile 500 ml flasks containing 300 ml 50% Potato Dextrose Broth (PDB). Flasks were placed into an incubator at 20°C and shook regularly to ensure even growth of *C. destructans*. After eight weeks, spore suspensions were produced by removing the mycelial matt and placing into 300 ml RO water followed by homogenising (MSE, England) and filtering through muslin (190 mm) to remove mycelium. Spore concentration was determined through serial dilution, as chains of spores made direct counts difficult to quantify the number of colony forming units (CFU) per ml of inoculum, and adjusted to 1 x 10^5 spores mL^{-1}. Parsnip seedlings were inoculated by pipetting 200 µl of spore suspension

at the base of the stem and wounded by piercing the base of the stem with a U-100 30 µl syringe needle (BD-Microfine, UK). Control (non-inoculated) seedlings were inoculated with 200 µl RO water and wounded as described above. The 308 module trays were placed onto individual 600 x 400 mm trays and watered from the bottom to prevent cross-contamination and dispersal of inoculum, temperature was maintained at 20°C with 16 h photoperiod. Seedlings were assessed for germination, development of disease symptoms and mortality twice weekly for two weeks. After two weeks, seedlings were individually harvested and scored for presence of brown root symptoms. One tray per isolate was arranged in a randomized design and the experiment was repeated four times.

4.2.3 Effect of temperature on *C. destructans* growth rate

Experiments were set up to determine the effect of temperature on the growth rate of *C. destructans* isolates on two different media using the same set of isolates selected for the pathogenicity experiments (Section 4.2.1, Table 4.1). Petri dishes containing 25 ml of PDA or Czapek Doz Agar (CDA) were centrally inoculated with a 5 mm mycelial plug taken from the actively growing edge of each *C. destructans* isolate. Plates were sealed with parafilm and placed into incubators at 5, 10, 15, 20 and 25°C, in darkness. A total of four replicate plates per isolate for each agar type was prepared and growth measurements of colony diameter at two perpendicular points were taken twice weekly for a period of 30 days. Following these experiments, *C. destructans* isolate CD28 was selected as a standard isolate to test the effect of additional temperatures, in order to perform more accurate parametric model fitting. A 5 mm mycelial plug was taken from the edge of an actively growing colony and placed into the centre of petri dishes containing 25 ml of PDA or CDA. Plates were sealed with parafilm and placed into incubators at 2.5, 17.5, 22.5 and 27.5°C in darkness. Four replicate plates per isolate for each agar type with twice weekly measurements of growth, as before, for a total of 30 days.

4.2.4 Effect of temperature on *C. destructans* spore production

Experiments were set up to determine spore production of *C. destructans* isolates at different temperatures on two different media using the same set of isolates as used in the pathogenicity experiments (Section 4.2.1, Table 4.1). A 5 mm mycelial plug taken from the edge of an actively growing plate was placed in to the centre of Petri dishes containing 25 ml synthetic nutrient-poor agar (SNA). Plates were placed into incubators at 5,10, 15, 20 and 25°C in darkness for 14 days. Once removed, 1 ml sterile RO water was added and spores gently removed using a sterile spreader. Spore suspensions were filtered through a 190 mm milk filter (Goat Nutrition Ltd, UK) to remove mycelium, and the number of spores determined using a haemocytometer. Four replicate plates were set up per isolate at each temperature, with ten haemocytometer counts per Petri dish.

4.2.5 Molecular characterisation of *C. destructans* isolates

C. destructans isolates were cultured on PDA at 20°C for 7 days to produce actively growing colonies. Three agar plugs of 5 mm^2 were taken from the leading edge, placed into Petri dishes containing 25 mL PDB, and incubated at 20°C for 7 days. Agar plugs were then removed and the mycelial mat washed three times in sterile RO water and blotted dry on tissue paper before being freeze-dried overnight. Genomic DNA was extracted from the freeze-dried mycelium using the DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. Isolate species identity was confirmed through PCR amplification and sequencing of the Internal Transcribed Spacer (*ITS*) ribosomal DNA and Translation Elongation Factor 1-α (*EF-1*α), RNA Polymerase II (*RpbII*) and Beta-Tubulin (*TUB2*) loci amplified and sequenced to investigate intra-species diversity. PCR was carried out in 20 µl reactions using a range of primers (Table 4.2). Reaction mixtures consisted of 10µl 1xREDTaq Ready Mix PCR reaction mix (Sigma-Aldrich, UK), 2 µl DNA template (10 ng), 1 µl primer template (0.4 µmol L^{-1}) and 6 µl purified water (Sigma-Aldrich, UK).

Thermocycling conditions for *ITS* are: one cycle of 2 min at 94°C, 40 cycles of 35 s at 94°C, 55 s at 61°C and 1 min at 72°C, followed by one cycle of 10 min at 72°C. For *EF-1*α: one cycle of 2 min at 94°C, 40 cycles of 35 s at 94°C, 55 s at 61°C and 1 min at 72°C, followed by one cycle of 10 min at 72°C. For Rpb2: one cycle of 4 min at 94°C, 34 cycles of 1 min at 94°C, 1 min of 55°C (1°C increase per cycle) and 1.5 min at 72°C, followed by one cycle of 10 min at 72°C. For TUB2: one cycle of 2 min at 94°C, 35 cycles of 45 s at 94°C, 30 s at 58°C and 1 min at 72°C, followed by one cycle of 7 min at 72°C. Specific primer pairs and PCR conditions are detailed in Table 4.2). Following PCR reaction, amplicons were visualized through gel electrophoresis using 1.5% agarose (Fischer Scientific) gels in 1 x tris-acetate-EDTA (TAE) buffer, stained with 2 µl / 100 ml of GelRed (Biotium, UK). PCR purification was then performed using the QIAquick PCR purification Kit (Qiagen); 5 µl DNA template and 5 µl 5pM sequencing primers (Table 4.2) were then submitted for sequencing by GATC (Konstanz, Germany).

Table 4.2 – Target gene loci, primers and annealing temperature used for PCR and sequencing of *C. destructans* isolates.

Genetic locus		Primer code	Primer sequence (5'-3')	Length (bp)	Annealing temp (°C)	Aplicon size (bp)	Source
ITS	Nuclear rDNA Internal transcribed spacer regions	ITS1	TCCGTAGGTGAACCTGCGG	19	61	520	Gardes & Bruns (1993)
		ITS4	TCCTCCGCTTATTGATATGC	20			White et al. (1990)
EF-1α	Translation Elongation Factor 1- α	EF595F	CGTGACTTCATCAAGAACATG	21	61	435	Kauserud & Schumacher (2001)
		EF1160R	CCGATCTTGTAGACGTCCTG	20			
Rpb2	RNA Polymerase II Second largest subunit	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	20	55*	857	Liu et al. (1999)
		bRPB2-7R2	ACYTGRTTRTGRTCNGGRAANGG	23			
TUB2	Partial β-Tubulin	T1	AACATGCGTGAGATTGTAAGT	21	58	600	O'Donnell & Cigelnik (1997)
		T2	TAGTGACCCTTGGCCCAGTTG	21			
		BT/2B	ACCCTCAGTGTAGTGACCCTTGGC	24		957	Glass & Donaldson (1995)

4.2.6 Pathogenicity of *M. acerina* isolates on parsnip roots

Experiments were set up to assess the pathogenicity of *M. acerina* isolates on freshly harvested parsnip roots from commercial parsnip growers. A total of seven isolates were isolated from diseased parsnip roots (Table 4.3). Parsnip roots (cv. Picador from) were mechanically harvested, washed with tap water, surface sterilized with 70% ethanol (v/v) and air-dried. Each root was checked for disease symptoms before the widest part of the root was inoculated with a 5 mm agar plug of *M. acerina* isolate from a ten-day-old actively growing colony on PDA, incubated at 20°C, in dark conditions. Parsnips were then incubated at 12°C on damp tissue in sealed 3 L plastic boxes and moist conditions maintained to encourage disease development by misting roots with RO water once a week. Six replicate parsnip roots were set up for each *M. acerina* isolate, and three repeat experiments were carried out. Photographs were taken weekly from three weeks post-inoculation and lesion areas measured using ImageJ (https://imagej.nih.gov/ij/).

Table 4.3 – Isolate number and origin of *M. acerina* isolates characterised in this study.

***M. acerina* isolate number**	**Isolation code**	**Site**	**Location**	**Country**
MA1	1	1	Lincolnshire	England
MA2	1	2	Spalding, Lincolnshire	England
MA3	1	3	Lincolnshire	England
MA4	1	3	Lincolnshire	England
MA5	1	2	Spalding, Lincolnshire	England
MA6	R9.3	2	Spalding, Lincolnshire	England
MA7	R14.6	2	Spalding, Lincolnshire	England

4.2.7 Pathogenicity of *M. acerina* isolates on parsnip seedlings

Experiments were set up assess the pathogenicity of different *M. acerina* isolates on parsnip seedlings in controlled environment experiments using the same set of isolates as used in the parsnip root pathogenicity experiments (Section 4.2.6, Table 4.3). *M. acerina* isolates were grown for 10 days at 20°C on PDA to produce actively growing colonies. Sterile flasks of 250 g sieved sharp sand and cornmeal (60 g) (Sigma-Aldrich, UK) were then inoculated with five mycelial plugs from the leading edge of each isolate and incubated at 20°C for eight weeks, with regular shaking to ensure even growth. Inoculum concentration was determined through serial dilution, as chains of spores made direct counts difficult, to quantify the number of colony forming units (CFU) per gram of inoculum. The inoculum was then mixed with sieved sharp sand to give a final concentration of 1×10^5 CFU g^{-1}. Thirty-five parsnip seeds (cv. Panache) were sown to a depth of 0.5 cm in 600 ml clear plastic boxes containing 250 g of the sand inoculum for each isolate, and 20 ml sterile RO water added to each box before sealing the lid. Boxes were placed in a controlled environment room at 20°C and 16 h photoperiod. Control (non-inoculated) seedlings were sown in 250 g sieved sharp sand only and 20 ml sterile RO water added as before. Seedlings were assessed for germination, development of disease symptoms and mortality twice weekly for 40 days. Forty days after sowing seedlings were harvested and scored for the presence of brown root symptoms. A total of four replicate boxes per isolate were arranged in a randomized block design and the experiment repeated four times.

4.2.8 Effect of temperature on *M. acerina* growth rate

Experiments were set up to determine the effect of temperature on mycelial growth of *M. acerina* isolates at different temperatures using the same set of isolates as used in the parsnip root pathogenicity experiments (Section 4.2.6, Table 4.3). Petri dishes containing 25 ml of PDA were centrally inoculated with a 5 mm mycelial plug taken from the actively growing edge of each *M. acerina* isolate. Plates were sealed with parafilm and placed into incubators at 5, 10, 15,

20 and 25°C, in darkness. A total of four replicate plates isolate were prepared and growth measurements of colony diameter at two perpendicular points were taken twice weekly for a period of 30 days. Following these experiments, *M. acerina* isolate MA2 was selected as a standard isolate to test the effect of additional temperatures, in order to perform more accurate parametric model fitting. A 5 mm mycelial plug was taken from the edge of an actively growing colony and placed into the centre of Petri dishes containing 25 ml of PDA. Plates were sealed with parafilm and placed into incubators at 2.5, 17.5, 22.5 and 27.5°C in darkness. Four replicate plates per isolate for each agar type with twice weekly measurements of growth, as before, for a total of 30 days.

4.2.9 Effect of temperature on *M. acerina* spore production

Experiments were set up to determine spore production of *M. acerina* isolates at 20 °C in the dark or under UV lighting using the same set of isolates as used in the parsnip pathogenicity experiments (Section 4.2.6, Table 4.3). A 5 mm mycelial plug taken from the edge of an actively growing plate was placed in to the centre of Petri dishes containing 25 ml PDA, and sealed with parafilm. Plates were placed into incubators at 20°C in darkness, or under 500 W / m^{-2} UV in an 8 h photoperiod. Once removed, 1 ml sterile RO water was added and spores gently removed using a sterile spreader. Spore suspensions were filtered through a 190 mm milk filter (Goat Nutrition Ltd, UK) to remove mycelium and the number of spores determined using a haemocytometer. Four replicate plates were set up per isolate for each light condition, with ten haemocytometer counts per Petri dish.

4.2.10 Genome sequencing and assembly of *M. acerina*

Genome sequencing and assembly of one *M. acerina* isolate, MA2 (Section 4.2.6, Table 4.3) was performed during this study primarily to design *M. acerina* specific primers for PCR amplification of housekeeping genes, as published universal fungal primer sequences (with the exception of *ITS*) were not

successful in amplifying DNA from *M. acerina* isolates. *M. acerina* isolate MA2 was grown on PDA at 20°C in dark conditions for 7 days to produce an actively growing colony. Three agar plugs of 5 mm were then taken from the leading edge, and placed into Petri dishes containing 25 mL potato dextrose broth (PBD), and incubated at 20°C in dark conditions for 7 days. Agar plugs were removed and the mycelial mat washed three times in sterile RO water and blotted dry on tissue paper before being freeze-dried overnight. Genomic DNA was extracted from the freeze-dried mycelium using the DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. Isolate species identity was confirmed through PCR amplification of the *ITS*. The genomic library was prepared using the Illumina Nextera DNA library preparation kit following the manufacturers protocol (Essex, UK), with the additional step of starting material was lightly sonicated on the Diagenode Bioruptor for 3 cycles at low power of 15 sec on, 180 sec off. The library was run on the Illumina Miseq using a 150bp paired end read (V2 cartridge kit) run. *De novo* assembly was carried out using the program Spades on the School of Life Sciences, Vettel Server, with a final N50 value of 9.4 kb.

4.2.11 Molecular characterisation of *M. acerina* isolates

Genomic DNA from *M. acerina* isolates (Section 4.2.6, Table 4.3) was prepared and extracted as described above (Section 4.2.10). Species identity was confirmed through PCR amplification and sequencing of the *ITS* ribosomal DNA with BLAST analysis. The Translation Elongation Factor 1-α (*EF-1α*), RNA Polymerase II (*RpbII*) and Beta-Tubulin (*TUB2*) loci were amplified and sequenced to investigate intra-species diversity. PCR was carried out in 20 µl reactions using a range of specifically designed primers (Table 4.4). Reaction mixtures consisted of 10 µl 1xREDTaq Ready Mix PCR reaction mix (Sigma-Aldrich, UK), 2 µl DNA template (10 ng), 1 µl primer template (0.4 µmol L^{-1}) and 6 µl purified water (Sigma-Aldrich, UK). Thermocycling conditions for *ITS* are: one cycle of 2 min at 94°C, 40 cycles of 35 s at 94°C, 55 s at 61°C and 1 min at 72°C, followed by one

cycle of 10 min at 72°C. For *EF-1*α: one cycle of 2 min at 94°C, 40 cycles of 35 s at 94°C, 55 s at 70°C and 1 min at 72°C, followed by one cycle of 10 min at 72°C. For Rpb2: one cycle of 4 min at 94°C, 34 cycles of 1 min at 94°C, 1 min of 60°C and 1.5 min at 72°C, followed by one cycle of 10 min at 72°C. For TUB2: one cycle of 2 min at 94°C, 35 cycles of 45 s at 94°C, 30 s at 57°C and 1 min at 72°C, followed by one cycle of 7 min at 72°C. Specific primer pairs and PCR conditions are detailed in Table 4.4). Following PCR reaction, amplicons were visualized using gel electrophoresis using 1.5% agarose (Fischer Scientific) gels in 1 x tris-acetate-EDTA buffer, stained with 2 µl/100ml of GelRed (Biotium, UK). PCR purification was then performed to extract DNA from the PCR reaction mixture using the QIAquick PCR purification Kit (Qiagen); 5 µl DNA template and 5 µl 5pM sequencing primers (Table 4.4) were then submitted for sequencing by GATC (Konstanz, Germany).

Table 4.4 – Target gene loci, primers and annealing temperature used for PCR and sequencing of *M. acerina* isolates.

Genetic locus		Primer code	Primer sequence (5'-3')	Length (bp)	Annealing temp (°C)	Aplicon size (bp)	Source
ITS	Nuclear rDNA Internal transcribed spacer regions	ITS1	TCCGTAGGTGAACCTGCGG	19	61	496	Gardes & Bruns (1993)
		ITS4	TCCTCCGCTTATTGATATGC	20			White et al. (1990)
EF-1α	Translation Elongation Factor 1- α	EF738	ATCATTGCCGCCGGTACTGG	20	70	880	Specifically designed for this study
		EF1754	AGCGGACTTGGTGACCTTGC	20			
Rpb2	RNA Polymerase II Second largest subunit	Rpb 35	TCGGTATCTGTGCCAGCATC	20	60	875	Specifically designed for this study
		Rpb 910	ATGGGCAATTGTCATTCGCG	20			
TUB2	Partial β-Tubulin	TUB 98	TCGAGCGCATGAGTGTTTAC	20	57	226	Specifically designed for this study
		TUB 324	CTTGGCCCAGTTGTTTCCAG	20			

4.2.12 Phylogenetic analysis of housekeeping gene sequence data for *C. destructans* and *M. acerina*

Housekeeping gene sequences obtained for all *C. destructans* and *M. acerina* isolates were trimmed and aligned using the ClustalW algorithm and refined using the MUSCLE algorithm implemented in MEGA v6 (Tamura et al., 2013). Two multi-locus concatenated phylogenetic trees, based on the *ITS* and housekeeping gene sequences, for each species was drawn, one using the 'Maximum Likelihood' and one the 'Neighbour Joining' option. Additional sequence data from GenBank was used to fully resolve the *Cylindrocarpon* (Table 4.5) and *Mycocentrospora* spp. (Table 4.6) phylogeny (www.ncbi.nlm.nih.gov).

Table 4.5 – *Cylindrocarpon* spp. isolates used in the phylogenetic analysis (in addition to *C. destructans* isolates described in Table 4.1) with their corresponding GenBank numbers.

Number	Species Name	Host	Origin	ITS Genbank Number
1	Cylindrocarpon destructans var. crassum	Tilia petiolaris(Silver Lime)	Germany	EF607078.1
2	Neonectria radicicola	Unknown	The Netherlands	HM364290.1
3	Ilyonectria radicicola	Bark - unknown	Venezuela	AF220969.1
4	Nectria radicicola	Apple (Malus domestica)	UK	AJ007351.1
5	Nectria radicicola	Apple (Malus domestica)	UK	AJ007353.1
6	Neonectria radicicola	Grapevine(Vitis vinifera)	Portugal	AJ875319.1
7	Neonectria radicicola	Grapevine(Vitis vinifera)	Portugal	AJ875320.1
8	Neonectria radicicola	Grapevine(Vitis vinifera)	Portugal	AJ875323.1
9	Neonectria radicicola	Grapevine(Vitis vinifera)	Portugal	AJ875333.1
10	Cylindrocarpon destructans var. destructans	Grapevine(Vitis vinifera)	Portugal	AM419059.1
11	Cylindrcarpon didymum	Pine (Pyrus sp.)	Canada	AY295303.1
12	Neonectria radicicola	American Ginseng (P. quinquefolius)	Canada	AY295309.1
13	Neonectria radicicola	Pine (Pyrus sp.)	Canada	AY295313.1
14	Cylindrocarpon cylindroides	Silver fir (Abies alba)	France	AY677261.1
15	Neonectria macrodidyma	Grapevine(Vitis vinifera)	South Africa	AM419071.1
16	Cylindrocarpon magnusianum	Soil	UK	AJ608955.1
17	Cylindrocarpon obtusisporum	Unknown	UK	AJ009263.1
18	Cylindrocarpon theobromicola	Cocoa tree (Theobroma cacao)	Papua New Guinea	EF607092.1
19	Cylindrocarpon cylindroides	Abies concolor (White Fir)	Netherlands	JF735312.1

Table 4.6 – *Mycocentrospora* spp. isolates used in the phylogenetic analysis (in addition to *M. acerina* isolates described in Table 4.3) with their corresponding GenBank numbers.

Number	Species Name	Host	Origin	ITS Genbank Number
1	Mycocentrospora acerina	Carum carvi var. dulce (Caraway)	The Netherlands	KC585407.1
2	Mycocentrospora acerina	Paeonia lactiflora (Peony)	Chile	KF015599.1
3	Mycocentrospora acerina	Panax notoginseng (Ginseng)	China	KP276614.1
4	Cerocospora cantuariensis	Humulus lupulus (Hop)	Austria	EU346862.1
5	Mycosphaerella delegaten	Eucalyptus camaldulensis (Eucalyptus)	Ethiopia	AB435070.1

4.2.13 Statistical analysis and modelling temperature effects on growth of *C. destructans* and *M. acerina* isolates

The size of *C. destructans* and *M. acerina* colonies were measured twice weekly for 30 days and, by visual inspection the period between day four and day eleven was selected as the optimum for calculation of the growth rate (mm day^{-1}) and further analysis (by day four, isolates had a consistent growth rate, whilst at day 11 maximum colony size had not yet been reached at any temperature). Data was assessed using a non-linear regression of biological temperature-dependent rate, based on absolute reaction-rate theory by Schoolfield *et al.* (1981). This model is derived based upon the assumptions that: 1) at all temperatures, the development rate of a poikilotherm population is determined by a single rate-controlling enzyme reaction and 2) this rate-controlling enzyme is reversibly denatured at high and low temperatures but maintains a constant total concentration independent of temperature. The model is derived from that of Sharpe & DeMichele (1977), who describe how the rate of a biological process is affected by temperature. Schoolfield *et al.* (1981) noted how the original model of Sharpe & DeMichele (1977) is ill-suited for linear regression techniques due to the high correlation between model parameters, so re-parameterised it in the following form:

$$r(T) = \frac{\rho_{(25°C)} \frac{T}{298} \exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2_L}} - \frac{1}{T}\right)\right] + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}, \quad (1)$$

where $r(T)$ is the mean growth rate at temperature T (time^{-1}), T is temperature in °K (298°K = 25°C), and R is the universal gas constant (1.987 cal deg^{-1} mol^{-1}). The other parameters are associated with the rate controlling enzyme reaction: $\rho_{(25°C)}$ is the development rate at 25°C assuming no enzyme inactivation (time^{-1}), $\Delta H_A^{\neq}$ is the enthalpy of inactivation of the reaction that is catalysed by the enzyme (cal mol^{-1}), $T_{1/2_L}$ is the temperature (°K) at which $\frac{1}{2}$ the enzyme is active and $\frac{1}{2}$ is inactive due to low temperatures, ΔH_L is the change in enthalpy

associated with low temperature inactivation of the enzyme (cal mol^{-1}), $T_{1/2_H}$ is the temperature (°K) at which the enzyme is $\frac{1}{2}$ active and $\frac{1}{2}$ high temperature inactive, ΔH_H is the change in enthalpy associated with high temperature inactivation of the enzyme (cal mol^{-1}). A reference temperature of 25°C was chosen by Schoolfield *et al.* (1981) although they mention other temperatures would have been equally valid and different temperatures might need to be selected for other organisms.

In order to perform the non-linear regression using equation (1), suitable starting values for the parameters were chosen following the suggestion of Schoolfield *et al.* (1981) and Davidson *et al.* (2003), i.e. by identifying the slopes of straight line regions of a curve fitted by eye to available data on an Arrhenius plot. Based on the data, in particular from CD28 and MA2 for which growth rate at additional temperatures was measured, the intermediate straight portion of the fitting curve on an Arrhenius plot suggests 15°C as a more reasonable reference temperature, rather than the 25°C used in (1). Therefore, the following modified form of equation (1) was used:

$$r(T) = \frac{\rho_{(15°C)}\ \frac{T}{288} \exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{288} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2_L}} - \frac{1}{T}\right)\right] + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}. \qquad (2)$$

When the data does not show evidence of reduced growth due to either high or low temperature inactivation, Schoolfield *et al.* (1981) suggest removing the exponential term of the denominator describing the corresponding reduction in growth. This eliminates two out of the six parameters that the data would be unable to identify. Given the data collected in this study always show evidence of inactivation due to high temperatures but not always due to low temperatures, and given our choice of reference temperature of 15°C, the four parameter reduced model used here takes the form:

$$r(T) = \frac{\rho_{(15°C)} \frac{T}{288} \exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{288} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}, \quad (3)$$

where parameters ΔH_L and $T_{1/2_L}$ have been removed.

Growth rates were calculated for all isolates of both *C. destructans* and *M. acerina* at five temperatures (5, 10, 15, 20 and 25°C), so using these data alone it was not possible to estimate six different parameters and therefore the four parameter model (3) was used. However, the additional measurements collected for the standard isolates *C. destructans* CD28 and *M. acerina* MA2 were sufficient to estimate six independent parameters for these isolates. Model fitting was carried out in MATLAB (Version 2015b) using the nlinfit function for non-linear regression curve fitting. Statistical analysis of growth rates for *C. destructans* and *M. acerina* isolates used a one-way analysis of variance (ANOVA) to determine isolate as a significant factor. A posthoc 'Tukey' test was carried out for each temperature (5, 10, 15, 20 and 25°C) to identify differences between isolates.

4.2.14 Statistical Analysis of *C. destructans* and *M. acerina* pathogenicity assays and spore production experiments

All statistical analysis was carried out using the software R (Version 0.98.945, R Development Core Team, 2014). For data from the *C. destructans* pathogenicity experiments on parsnip roots, lesion area (cm^2) was loge-transformed to satisfy the requirements of homogeneity of variance and reduce the influence of residuals. Transformed data were then analysed using a one-way ANOVA and the 5% LSD value calculated. The percentage diseased seedlings data (seedling death, plus the presence of a brown root symptom at harvest) from the CD pathogenicity experiments on parsnip seedling were log transformed and analysed using a general linear model (GLM); the posthoc test 'Tukey' was applied to determine difference between isolates at each temperature. For

experiments examining spore production by *C. destructans*, mean log spore count density (spores mm^{-2}) was plotted against temperature. Data were then analysed using a one-way ANOVA, and the posthoc test 'Tukey' applied to determine difference between isolates at each temperature.

Data from the *M. acerina* pathogenicity experiments on parsnip roots lesion area (mm^2) were analysed using a one-way ANOVA. The percentage diseased seedlings data (seedling death, plus the presence of a brown root symptom at harvest) from the CD pathogenicity experiments on parsnip seedling were log transformed, and analysed using a one-way ANOVA and the posthoc test 'Tukey'. For experiments examining spore production by *M. acerina*, mean log spore count density (spores mm^{-2}) was plotted against temperature. Data was then analysed using a one-way ANOVA, and the posthoc test 'Tukey' applied to determine difference between isolates at each temperature.

4.3 Results

4.3.1 Pathogenicity of *C. destructans* isolates on parsnip roots

All 16 *C. destructans* isolates caused lesions on parsnip roots following inoculation (Fig 4.1), with mean lesion area ranging from 0.4 cm^2 to 2.0 cm^2 after 50 days (Fig. 4.2). The ANOVA indicated a significant effect of *C. destructans* isolate ($p<0.05$). Isolates separated into seven groups (Table 4.7), based upon from each other. The greatest lesion area (cm^2) was observed for isolates CD31 and CD1, which were significantly different ($p<0.05$) from all isolates except for CD30 and CD10. Isolate CD24 had the smallest lesion area, which was significantly different ($p<0.05$) from all the other isolates except CD19 and CD23.

Figure 4.1 – Symptoms of *C. destructans* inoculated parsnip (cv. Javelin) roots. A) *C. destructans* isolate CD10 (more aggressive). B) *C. destructans* isolate CD23 (less pathogenic).

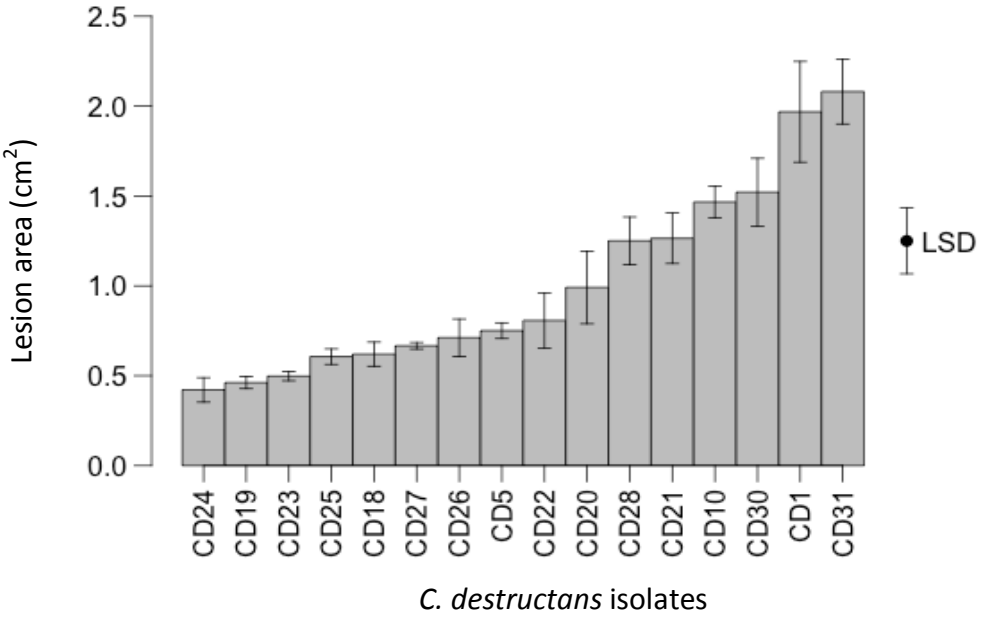


Figure 4.2 – Lesion area (cm^2) for 16 *C. destructans* isolates on parsnip roots (cv. Javelin). Error bars are SEM from four replicates. LSD indicated at 5% level.

Table 4.7 – Summary of log_e-transformed means and 5% LSD value for all *C. destructans* isolates from parsnip lesion root pathogenicity assay. The degrees of freedom (d.f.) were 15.

Number	Isolate	Log_e-transformed means	5% LSD
1	CD31	0.69224408	A
2	CD1	0.67481368	A
3	CD30	0.35767767	AB
4	CD10	0.34556075	ABC
5	CD28	0.21530344	BC
6	CD21	0.21071123	BC
7	CD20	-0.01595975	CD
8	CD22	-0.21743585	DE
9	CD5	-0.3282365	DE
10	CD26	-0.35030179	DE
11	CD27	-0.42973310	EF
12	CD18	-0.49008021	EF
13	CD25	-0.50853839	EF
14	CD23	-0.71645240	FG
15	C19	-0.77473880	FG
16	CD24	-0.89063320	G
d.f.	15		
5% LSD	-0.375		

4.3.2 Pathogenicity of *C. destructans* isolates on parsnip seedlings

All 16 *C. destructans* isolates caused brown root symptoms to develop on parsnip seedlings, with the mean number of seedlings infected ranging from 10% (CD23) to 89% (CD21), with no significant disease for the control treatment. The Shapiro-Wilk test for normality ($p>0.05$) indicated a normal distribution within the data, subsequent GLM analysis showed a significant effect of isolate ($p<0.05$) on seedling disease percentage, and a multicomparison 'Tukey' posthoc test (Table 4.6) identified a number of different groups. All *C. destructans* isolates with the exception of CD24 (due to the larger SEM of this isolate) were significantly different ($p<0.01$) from the control. Isolates CD23, CD18, CD30, CD31, CD10, CD1 and CD20 were significantly different only from the control, while isolates CD26, CD22, CD5, CD27, CD25, CD19, CD25, CD28 and CD21 were significantly different from at least two other isolates. The four most pathogenic isolates, CD19, CD25, CD28 and CD21 are also markedly different from the largest number of isolates (9, 9, 8 and 7 isolates, respectively) with a significance of $p<0.001$.

Incomparing the virulence of C. destructans isolates from both the parsnip root and parsnip seedling assays there is little correlation between isolates. Results show isolates displaying higher virulence in the root pathogenicity assay display a range of higher, intermediate and low viulence in the seedling assay. As such no correlation can be identified between isolates in comparing virulence in parsnip roots and seedlings.

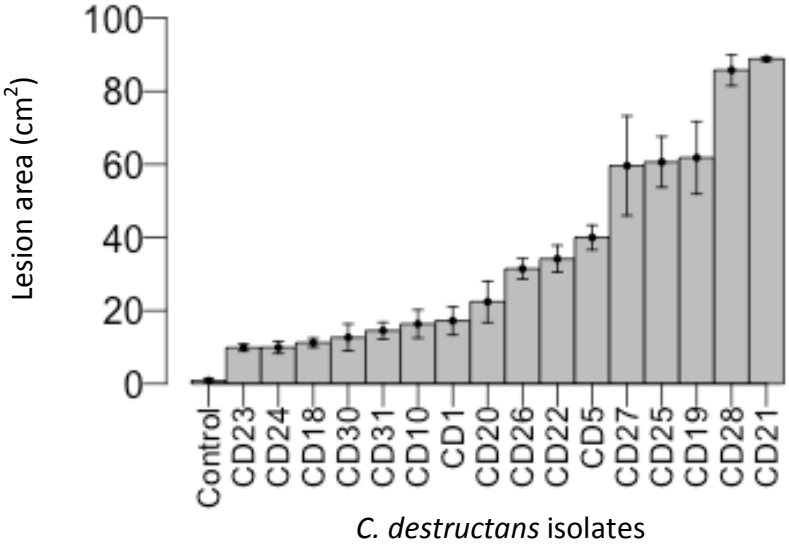
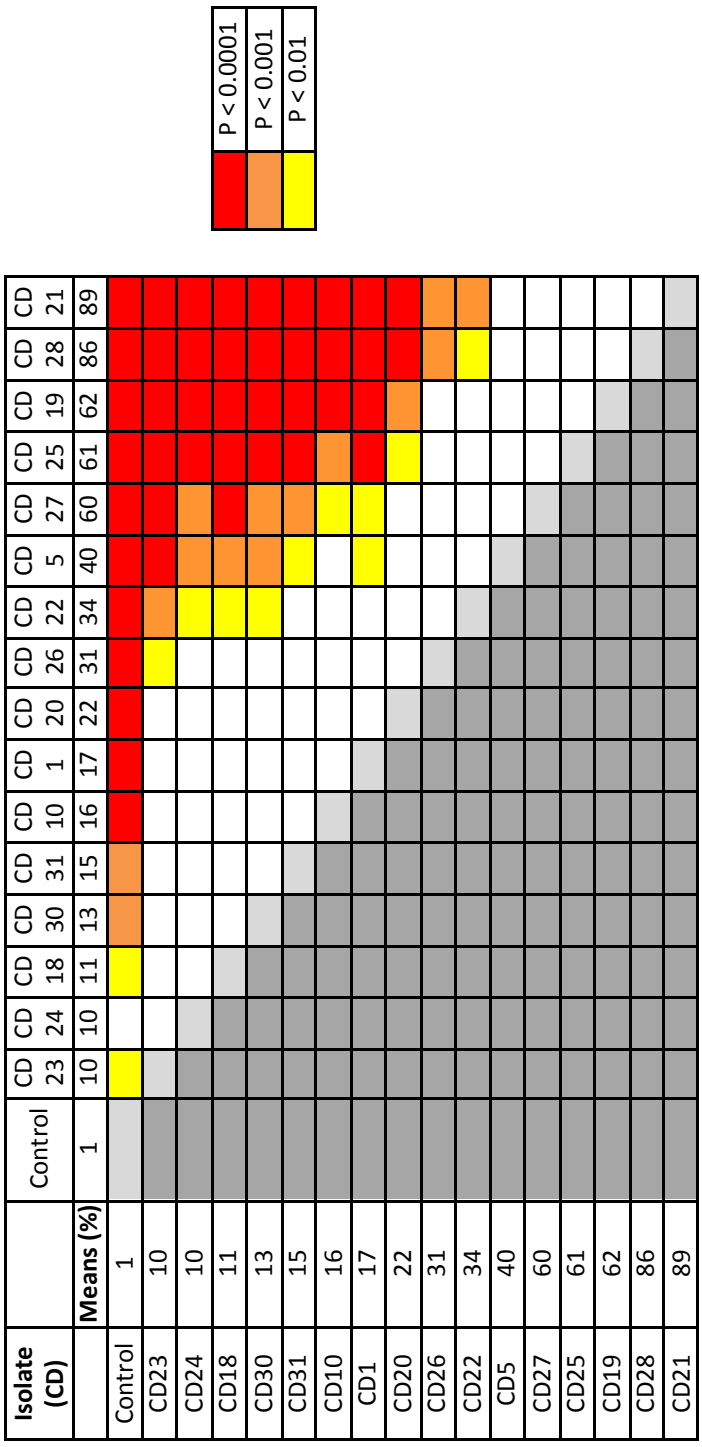


Figure 4.3 – Percentage parsnip (cv. Panache) seedlings affected (seedling death / brown root symptoms) by 16 *C. destructans* isolates. Error bars show SEM from four replicate experiments.

Table 4.8 – Summary of 'Tukey' multicomparison posthoc test for significance for all *C. destructans* isolates from parsnip (cv. Panache) seedling inoculation. Yellow colour indicates a significant difference of p<0.01, orange of p<0.001 and red of p<0.0001.



4.3.3 Effect of temperature on *C. destructans* growth rate

Rate of mycelial growth for different *C. destructans* isolates was assessed over five temperatures (5, 10, 15, 20 and 25°C) on two different media types (PDA and CDA). All isolates grew at all temperatures tested with a general trend of increasing growth rate with increasing temperature. The growth rates for isolates on both agar types were very similar, so a combined average is reported here. The minimum average rate of growth for all isolates across both agar types was at 5°C (1.34 mm day^{-1}), with values ranging from 0.46 mm day^{-1} to 2.14 mm day^{-1}, and the maximum average growth rate at 20°C (5.68 mm day^{-1}), with values ranging from 3.55 mm day^{-1} to 7.84 mm day^{-1} (Fig. 4.3). The growth rate at 20°C displayed the smallest variation between isolates, with isolate growth rates at 5°C and 25°C displaying the greatest variation (from 2.14 mm day^{-1} to 0.46 mm day^{-1} and from 0.88 mm day^{-1} to 6.19 mm day^{-1}, respectively).

Some *C. destructans* isolates, such as CD5, CD30 and CD31, displayed consistently lower growth rates compared to other isolates at most temperatures across both agar types, whilst isolates CD1 and CD22 displayed consistently higher growth rates. Some isolates such as CD19, CD20, CD24 and CD28 ranked consistently in the middle range for all temperatures assessed. The ANOVA revealed that *C. destructans* isolate has a significant effect ($p<0.05$) on growth rate, but agar type had no significant effect ($p>0.05$) on growth rate. A posthoc 'Tukey' analysis was carried out to perform pairwise comparisons between isolates at each temperature, for both agar types. Overall, this analysis showed a greater number of significant differences between isolates grown on CDA than on PDA. However, *C. destructans* isolates CD22 (consistently low growth rate), CD30 and CD31 (consistently high growth rates) were significantly different ($p<0.05$) from the largest number of other isolates across all temperatures and both agar types. 'Tukey' outputs for comparison between all isolate at all temperatures can be found in the appendix (Section 4.5.1).

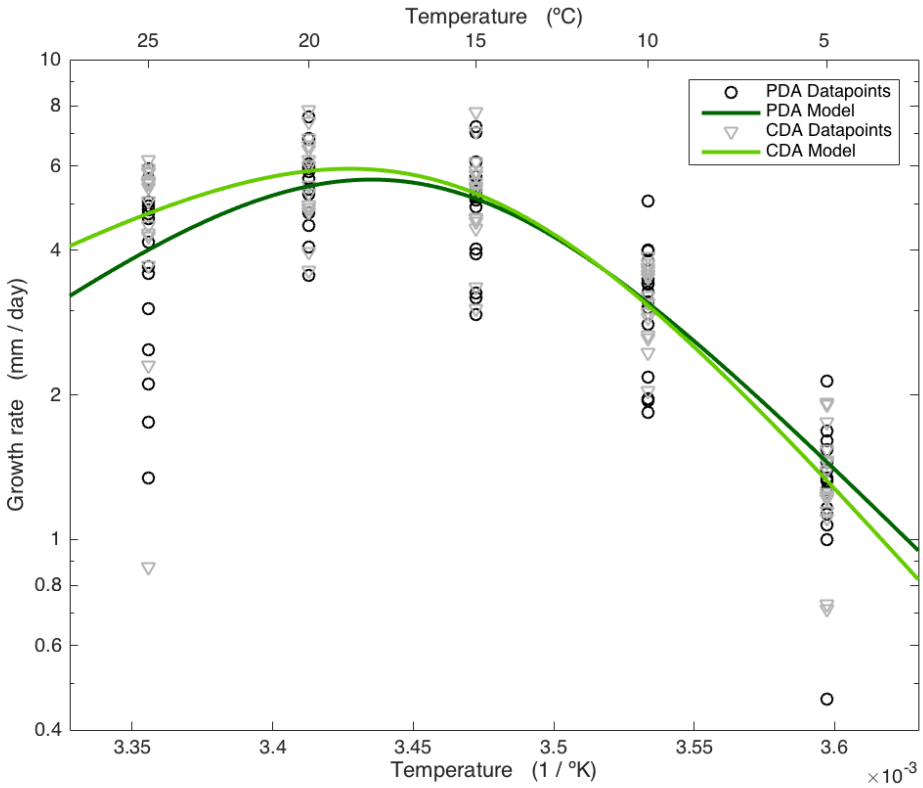


Figure 4.4 – Model predictions for the effect of temperature on the growth rate of *C. destructans* isolates (Table 4.1). Light green line represents fitted Arrhenius curve obtained using equation (3) for all *C. destructans* isolates grown on CDA, with grey triangles represent individual isolates. Dark green line represent fitted Arrhenius curve obtained using equation (3) for all *C. destructans* isolates grown on PDA, with black circles representing individual isolates. Data points are the mean growth rate of four replicates.

In addition to the five temperatures tested above, *C. destructans* isolate CD28 was selected as a standard isolate for *C. destructans* and assessed across a further four temperatures of 2.5, 17.5, 22.5 and 27.5°C (Fig 4.5). On both agar types, the isolate displayed the lowest rates of growth at both 2.5°C (PDA – 1.18 mm day^{-1} and CDA – 1.43 mm day^{-1}) and 27.5°C (PDA – 1.18 mm day^{-1} and CDA – 1.50 mm day^{-1}), with a maximum growth rate at 22.5°C (PDA – 6.71 mm day^{-1} and CDA – 6.46 mm day^{-1}).

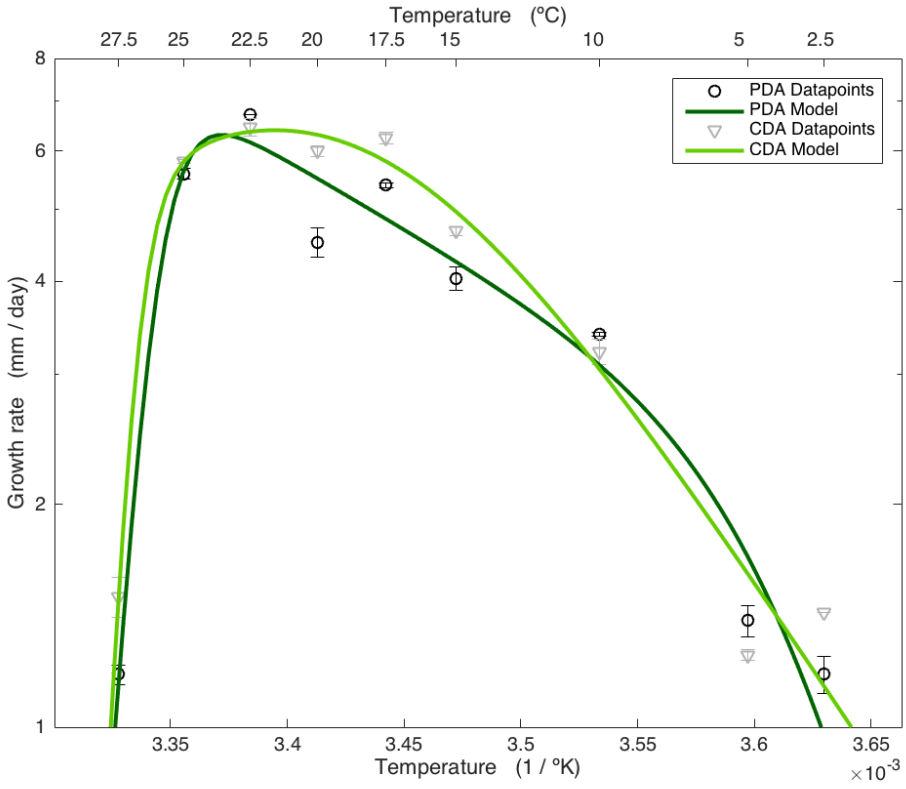


Figure 4.5 – Model predictions for the effect of temperature on the growth rate of *C. destructans* isolate CD28 (Table 4.1). Light green line represent fitted Arrhenius curve obtained using equation (2) for CD28 grown on CDA, with grey triangles representing mean data-points. Dark green line represent fitted Arrhenius curve obtained using equation (2) for CD28 grown on PDA, with black circles representing individual mean data-points. Individual data-points represent mean growth rate of four replicates; error bars show SEM.

The parameter estimates from the model fitting (Table 4.9) vary significantly between the four parameter model (equation (3)) fitted to all the C. destructans isolates and the six parameter model (equation (2)) fitted to the standard C. destructans isolate, CD28. As an example $\rho_{(15°C)}$ the estimated rate of growth at 15°C (assuming no enzyme inactivation), are 8.17 mm day^{-1} for PDA and 9.48 mm day^{-1} for CDA for all the C. destructans isolates and is biologically less reasonable than the 4.31 mm day^{-1} estimated for C. destructans isolate CD28. These differences can be attributed to two main reasons. First, the growth rate of *C. destructans* isolate CD28 was measured at 2.5°C and 27.5°C; at these temperatures, very low growth rates were recorded in contrast to the higher growth rates at intermediate temperatures. This enabled better estimates for

the temperatures at which half high enzyme inactivation and half low enzyme inactivation occur, leading to biologically plausible parameter estimates. Second, from the ANOVA analysis described above, significant differences were identified between isolates at all temperatures. This made it challenging for the four-parameter model to accurately capture such a high level of variation. In addition the parameter estimates for CD28 grown on CDA are also biologically unreasonable, primarily due to the rate of growth at 2.5°C (1.43 mm day^{-1}) appearing higher than that of 5°C (1.25 mm day^{-1}), instead of an expected decrease in growth rate at decreasing temperatures (as for CD28 grown on PDA). This makes the fitting procedure more challenging, resulting in inaccurate parameter estimates.

Table 4.9 – Summary of parameter estimates for the effect of temperature on the rate of growth of *C. destructans* isolates and standard isolate CD28. Estimations based on the modified Schoolfield et al. (1981) equations (2) and (3) as described in the text.

	$\boldsymbol{\rho_{(15°C)}}$ **mm day^{-1}**	$\boldsymbol{\Delta H_A^{\neq}}$ **cal mol^{-1}**	$\boldsymbol{\Delta H_H}$ **cal mol^{-1}**	$\boldsymbol{T_{1/2_H}}$ **(°C)**	$\boldsymbol{\Delta H_L}$ **cal mol^{-1}**	$\boldsymbol{T_{1/2_L}}$ **(°C)**
C. destructans - PDA	8.1708	26436	46263	16.874	-	-
C. destructans - CDA	9.475	29957	44504	15.824	-	-
C. destructans CD28 - PDA	4.3131	7712.7	$2.27x10^5$	26.107	-56729	3.1793
C. destructans CD28 - CDA	18.867	-15254	$2.90x10^5$	26.793	-36916	19.666

4.3.4 Effect of temperature on *C. destructans* spore production

The number of spores produced by C. destructans isolates was assessed over five temperatures (5, 10, 15, 20 and 25°C) with the total spore production for each isolate expressed as spore density (spores mm^{-2}), to control for the variation in colony size observed at different temperatures. The general trend of the data suggested that spore production of all isolates remained constant across all the temperatures tested (Fig. 4.6). Spore densities ranged from 0.01 to 534.80 across all temperatures, and showed a similarly high range of values at 5°C and 25°C, with 0.01 – 244.33 spores mm^{-2} and 0.1 – 453.52 spores mm^{-2} respectively (Fig 4.6).

The ANOVA showed a significant effect of *C. destructans* isolate ($p<0.001$) on spore production; the post-hoc 'Tukey' analysis identified significant interactions between isolates at each temperature. The number of significant interactions between isolates increased with temperature, with C. destructans isolates CD1, CD5 and CD22 displaying consistently low densities while other, such as CD23 and CD24 display consistently high densities across most temperatures. Full results of the 'Tukey' analysis can be found in the appendix.

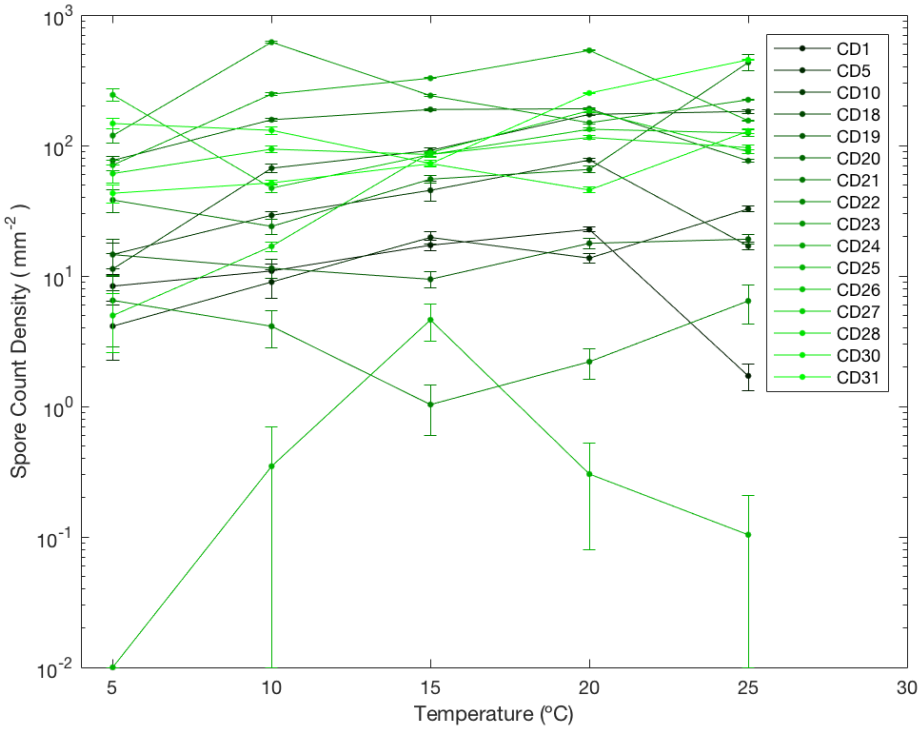


Figure 4.6 – Log_{10} spore density (spores mm^{-2}) at five temperatures for *C. destructans* isolates (Table 4.1) grown on CDA for 10 days. Data-points represent mean spore count of four replicates, error bars show SEM.

4.3.5 Molecular characterisation of *C. destructans* isolates

Following confirmation of *C. destructans* species identity through *ITS* sequencing and BLAST analysis, sequence data for *ITS*, and for the housekeeping genes *EF-1α*, *Rpb-II* and *TUB2* were assembled and a phylogenetic analysis carried out to identify intra-species diversity.

The maximum likelihood and neighbour joining analyses for the ITS locus included the 16 *C. destructans* isolates and 14 additional *Cylindrocarpon* and related spp. sequences from GenBank. Both the maximum likelihood and neighbour joining analyses separated the *Cylindrocarpon* spp. isolates into three major clades of 26, 4 and 5 isolates respectively (Fig 4.7 and Fig 4.8). Each of the three clades is further divided into genetic clusters (I, II, III, IV and V, Fig 4.7 and Fig 4.8), none of which appears to be related to host, or geographic location. For

example, isolates in the same clade were found from Lincolnshire, Scotland, Canada and Portugal, and isolated from parsnip, grapevine and pine. Genetic clusters I and II, contain only *C. destructans* isolates, where *Neonectria radicicola*, *Nectria radicicola* and *Ilyonectria* are anamorphs of *C. destructans*. Whilst clusters III, IV, V and VI contain multiple *Cylindrocarpon spp.* from a range of hosts, including parsnip, grapevine, apple, ginseng and fir. For example cluster III contains the species *Neonectria radicicola*, *Neonectria macrodidyma* and *Nectria radicicola* from apple and grapevine hosts from the UK and Portugal respectively, again suggesting that clustering is not related to host or location. The 16 *C. destructans* isolates from parsnip are in cluster I (10 isolates), cluster V (2 isolates) and cluster VI (4 isolates). The two isolates in cluster V, appear to be most closely related to *C. theobromicola* and *C. obtusisporum*, whilst the 4 isolates in cluster VI appear to be most closely related to *C. cylindroides*, despite all *Cylindrocarpon* isolates off parsnip hosts being confirmed as *C. destructans* through BLAST analysis. Bootstrap values on both the maximum likelihood and neighbour joining analyses ranged from 24 to 99%, with the majority above 75% in the maximum likelihood analysis and above 50% in the neighbour joining analysis.

Concatenated alignments of the housekeeping genes *ITS*, *EF-1α*, *Rpb-II*, and *TUB2* were analysed using both maximum likelihood and neighbour joining analyses (Fig 4.9 and Fig 4.10). Both phylogenies were very similar and divided the *C. destructans* isolates into two clades, with each clade further sub-divided into different genetic clusters. None of the isolates within each of the clades appear to be related by geographic location, and as all isolates are from parsnip hosts, and are split across different genetic cluster, it is unlikely they are related by host. The bootstrap values for branches on both the maximum likelihood and neighbour joining analysis are all above 66%, with the majority above 90% indicating good support for branches.

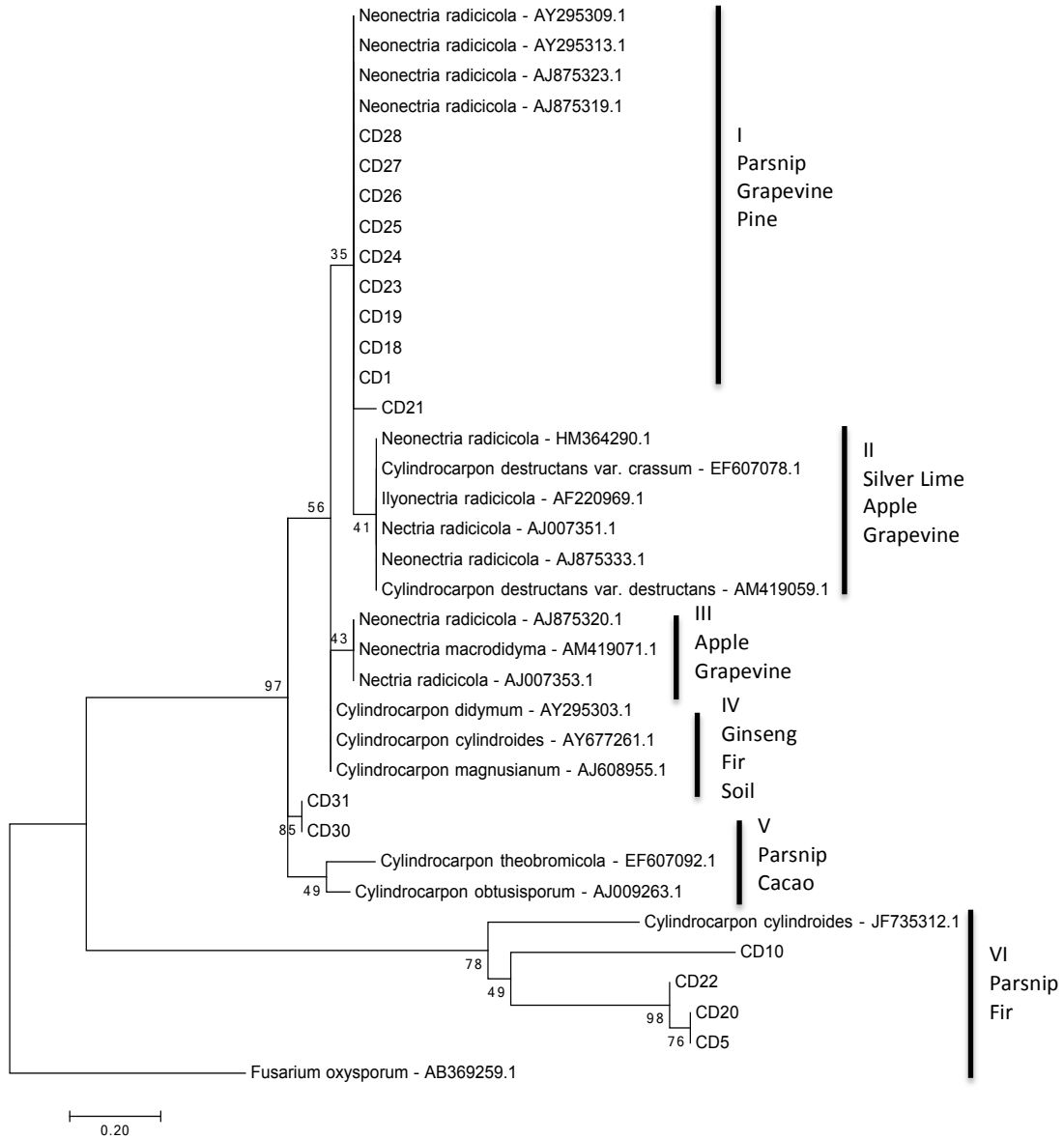


Figure 4.7 – Maximum Likelihood phylogenetic tree for *C. destructans* isolates based on an alignment of the *internal transcribed spacer (ITS)* sequences. Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site. The tree is rooted with *Fusarium oxysporum* (AB369259.1), with *C. theobromicola*, *C. cylindroides*, *C. obtusisporum*, *C. magnusianum* and *C. didymium* included as related species and *I. radicicola* and *N. radicicola* (Cabral *et al.*, 2012) a synonym of *C. destructans*.

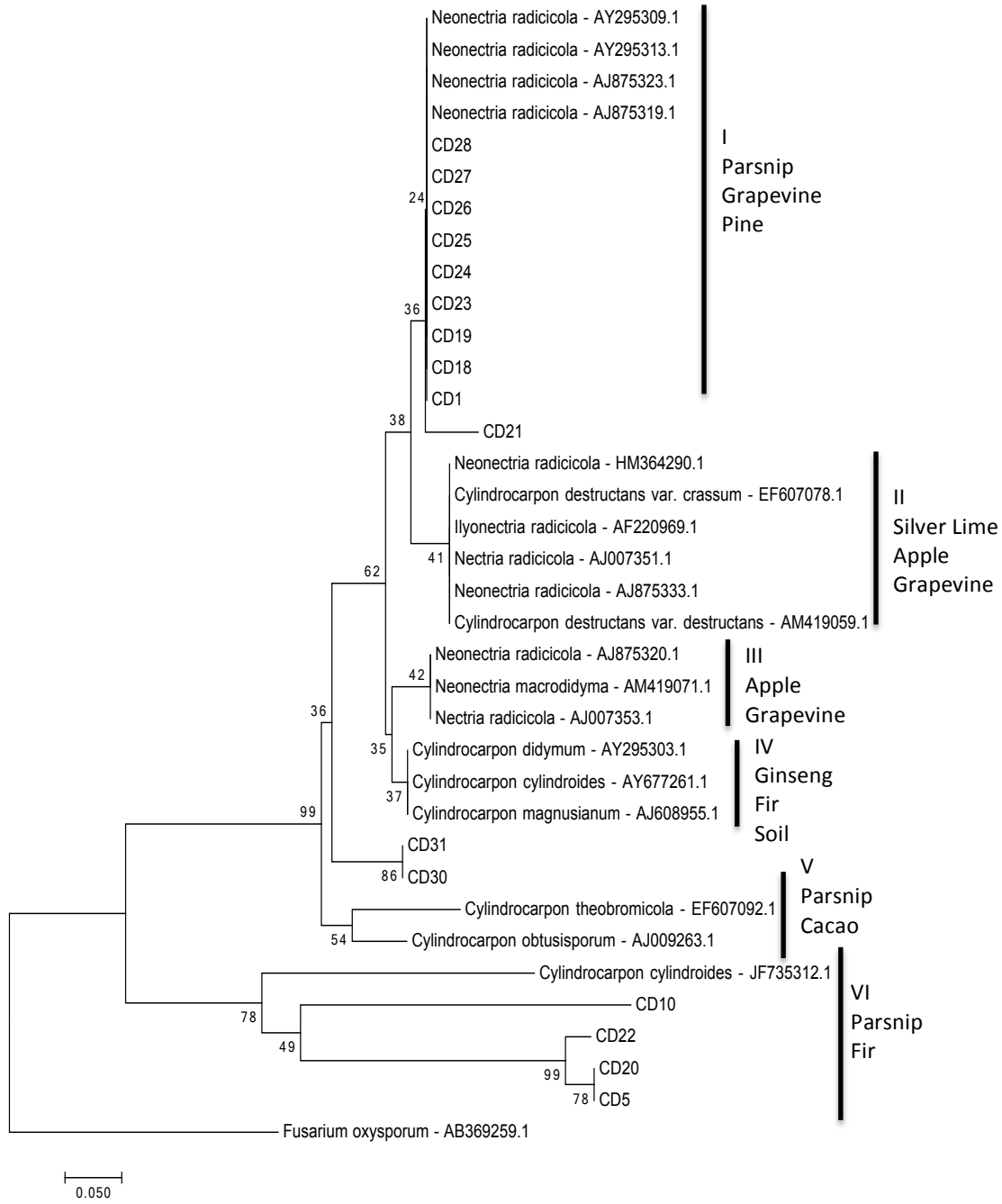


Figure 4.8 – Neighbour Joining phylogenetic tree for *C. destructans* isolates based on an alignment of the internal transcribed spacer (*ITS*) sequences. Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site. The tree is rooted with *Fusarium oxysporum* (AB369259.1), with *C. theobromicola*, *C. cylindroides*, *C. obtusisporum*, *C. magnusianum* and *C. didymum* included as related species and *I. radicicola* and *N. radicicola* (Cabral *et al.*, 2012) a synonym of *C. destructans*.

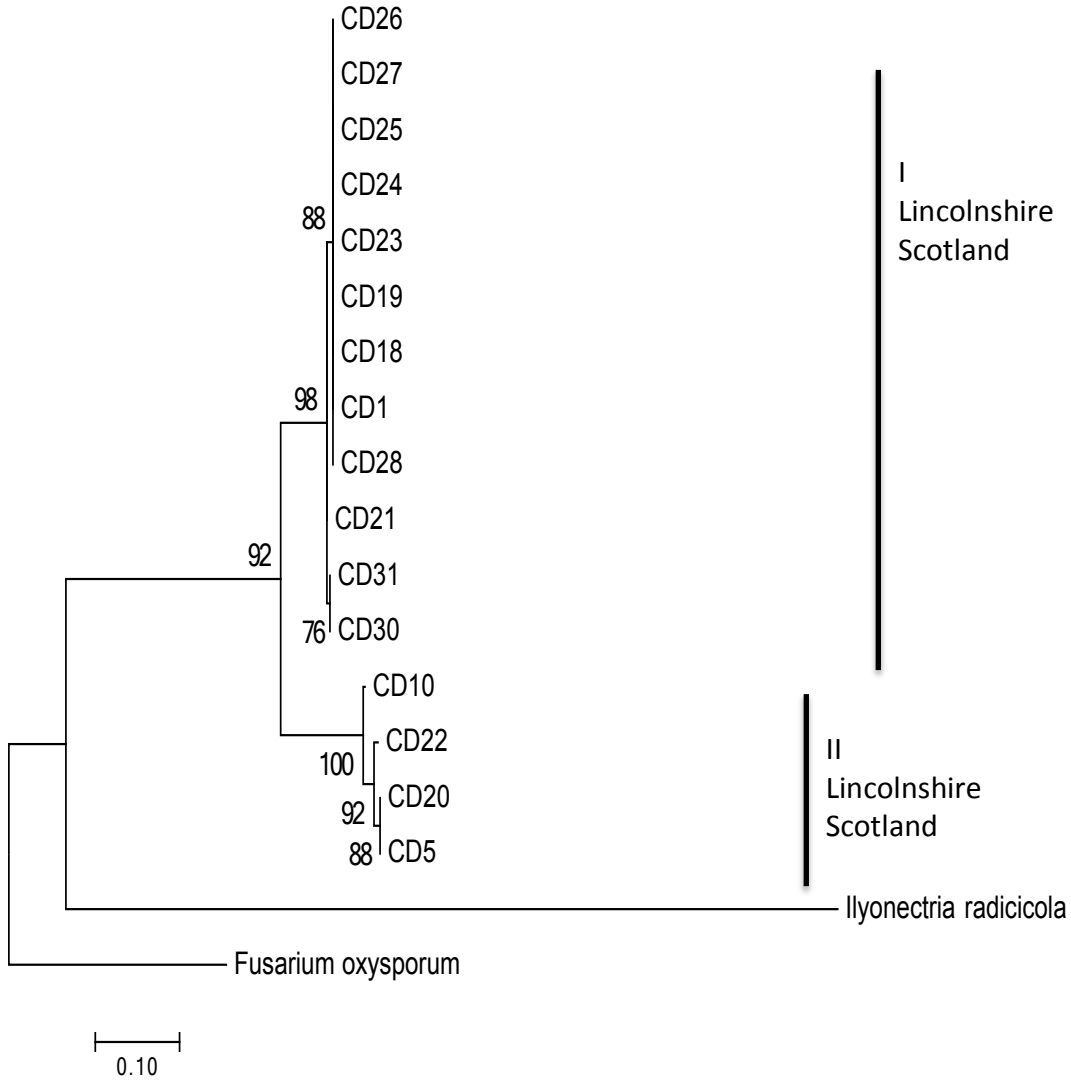


Figure 4.9 – Maximum Likelihhood phylogenetic tree for *C. destructans* isolates based on an alignment of the internal transcribed spacer (*ITS*), RNA polymerase II (*rpb-II*), translation elongation factor (*EF-1α*) and Beta-tubulin (*TUB2*). Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.10 substitutions per site. The tree is rooted through *Fusarium oxysporum* (AB369259.1) with *I. radicicola* (Cabral *et al.*, 2012) an anamorph of *C. destructans*.

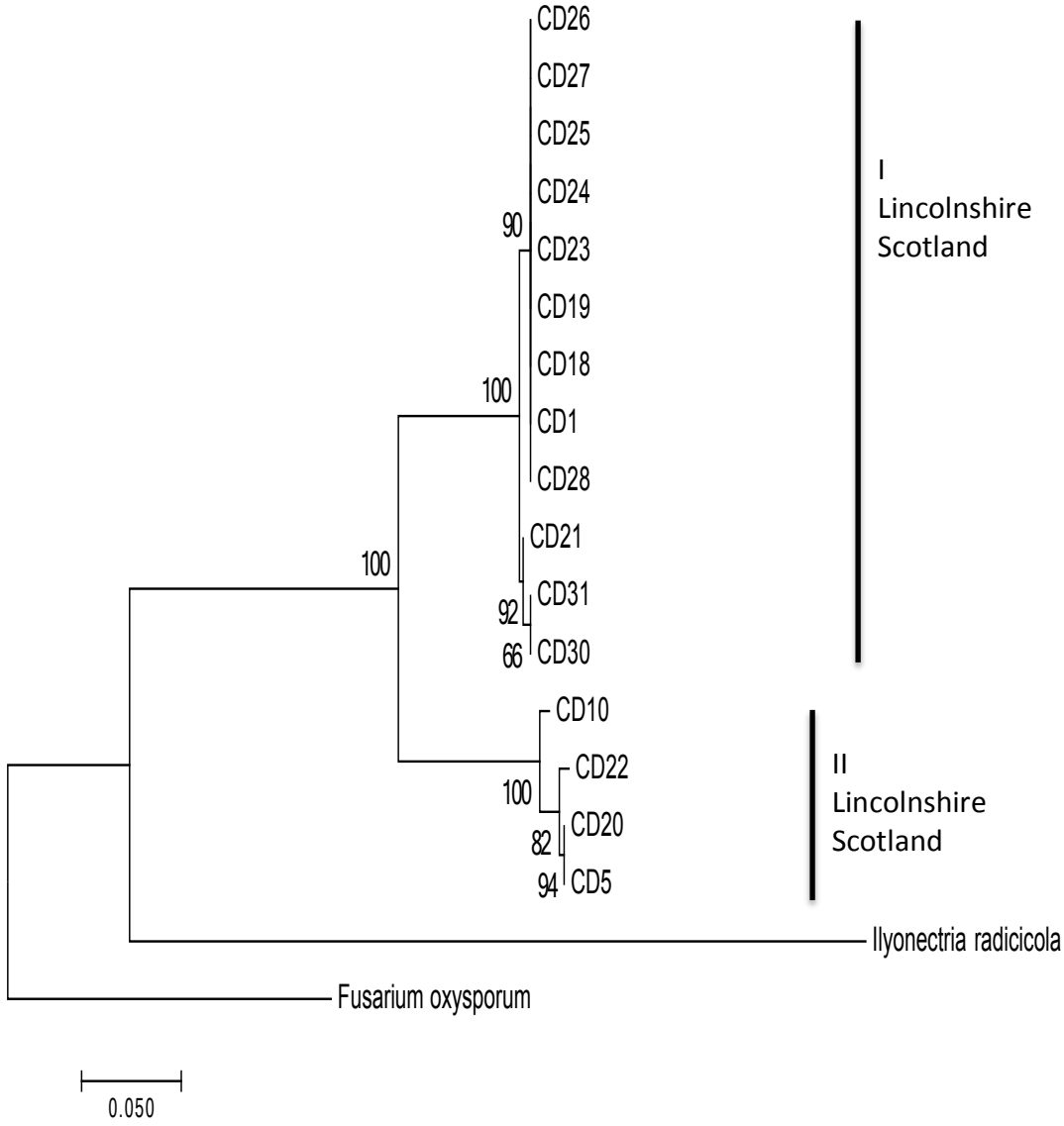


Figure 4.10 – Neighbour Joining phylogenetic tree for *C. destructans* isolates based on an alignment of the internal transcribed spacer (*ITS*), RNA polymerase II (*rpb-II*), translation elongation factor (*EF-1α*) and Beta-tubulin (*TUB2*). Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site.

4.3.6 Pathogenicity of *M. acerina* isolates on parsnip roots

Following inoculation with *M. acerina* isolates all parsnip roots developed lesions (Fig 4.11), with mean lesion area ranging from 3.0 cm^2 to 8.0 cm^2 after 50 days (Fig 4.12). The ANOVA of the lesion area (cm^2) indicates no significant difference between isolates ($p>0.05$) due to the large SEM, so no posthoc or multicomparison test was required. There is, however, a marked different between MA7 and MA2, the isolates showing the lowest and highest lesion areas respectively.

A B

Figure 4.11 – Symptoms of *M. acerina* inoculated parsnip (cv. Picador) roots. A) *M. acerina* isolate MA2 (more aggressive). B) *M. acerina* isolate MA7 (less pathogenic).

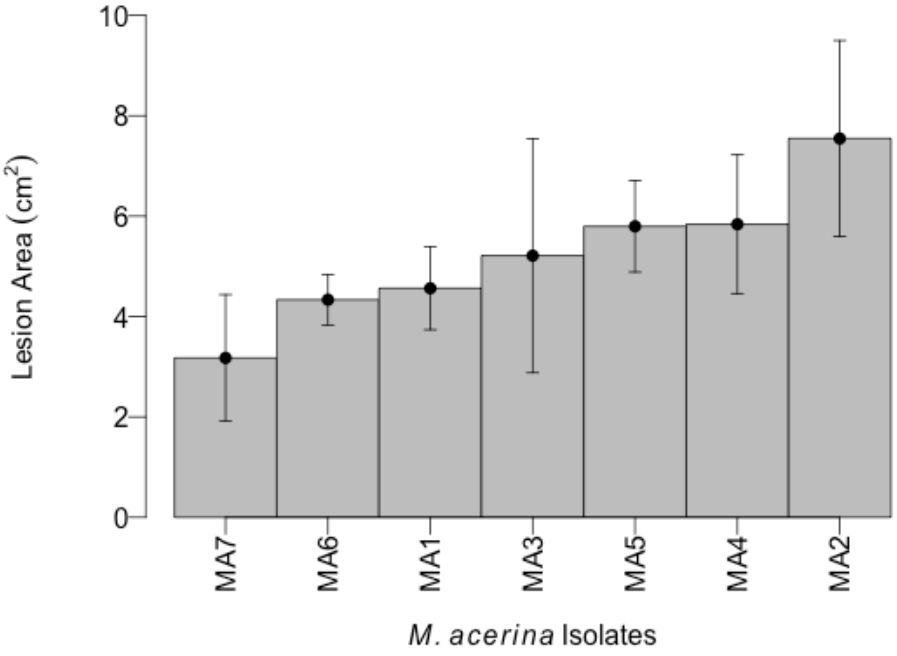


Figure 4.12 – Lesion area (cm^2) for 7 *M. acerina* isolates on parsnip roots (cv. Picador). Error bars are SEM from four replicates.

4.3.7 Pathogenicity of *M. acerina* isolates on parsnip seedlings

All seven *M. acerina* isolated caused brown root symptoms and damping off in parsnip seedlings, with no symptoms present in the control group. The mean number of seedling infected was 2.5% in the control group and a range of 85% to 96% in seedling grown in *M. acerina* inoculum (Fig 4.13). The ANOVA of seedling disease (%) indicates a significant effect ($p<0.05$) of *M. acerina* isolates on seedling disease. The posthoc muticomparison 'Tukey' test was carried out to highlight significant differences between individual isolates. All *M. acerina* isolates were significantly different ($p<0.0001$) to the control, but there were no significant difference ($p>0.05$) between any *M. acerina* isolates.

In comparing results from the M. acerina parsnip root and seedling pathogenicity assay, there is little correlation. Isolates displaying higher virulence in the root

pathogenicity assay, display a range of higher, low and intermediate virulence in the seedling assay. Therefore, there is no clear correlation between the virulence of M. acerina isolates across the parsnip root and seedling assays.

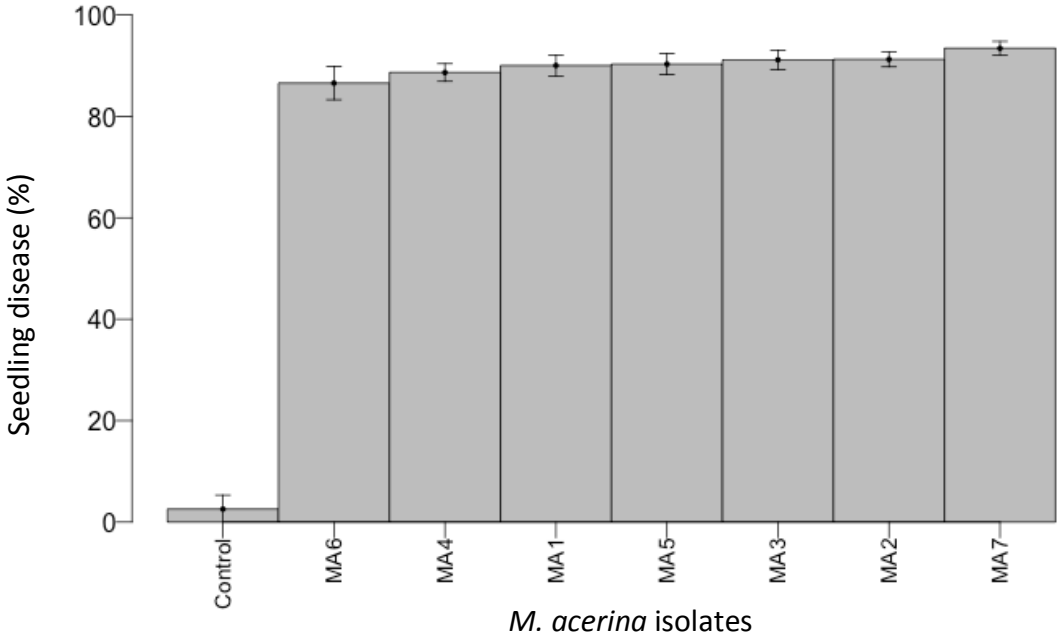


Figure 4.13 – Percentage parsnip (cv. Panache) seedlings affected (seedling death, plus brown root symtpoms) by 7 *M. acerina* isolates. Error bars show SEM from four replicate experiments.

4.3.8 Effect of temperature on *M. acerina* growth rate

The growth rate of *M. acerina* isolates was assessed over five temperatures (5, 10, 15, 20 and 25°C). All isolates grew across all temperatures with a general trend of increased growth rate with increased temperature. The lowest rate of growth for all isolates was at 25°C (0.43 mm day^{-1}), with the maximum growth rate at 20°C (5.57 mm day^{-1}) and the greatest range of growth rate between isolates at 25°C (-0.14 mm day^{-1} to 2.13 mm day^{-1}).

The ANOVA of growth rate for *M. acerina* showed a significant effect of isolate ($p<0.001$). The temperature where isolates were the most significant was 25°C, with MA2 and MA5 significantly different ($p<0.001$) from all other isolates. Significance between isolates was present at 5°C, with MA2 and MA5 showing significance to all other isolates ($P<0.05$ and $P<0.01$). 'Tukey' outputs for comparison between all isolates at all temperatures can be found in the appendix (Section 4.5.3).

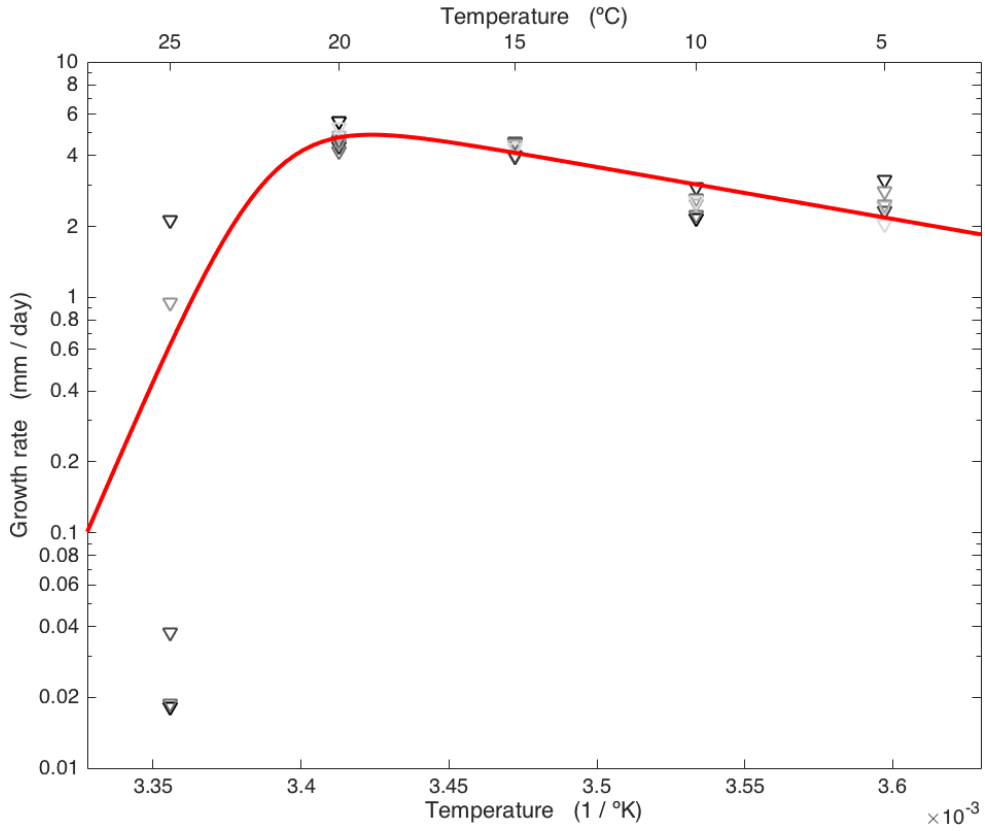


Figure 4.14 – Model predictions for the effect of temperature on the growth rate of *M. acerina* isolates (Table 4.3) on an Arrhenius plot. Red line represents fitted curve obtained using equation (3) with triangles representing mean growth of each isolate with four replicates.

In addition to the five temperatures investigated above, MA2 was selected as a standard isolate for *M. acerina* and assessed across a further four temperatures (2.5, 17.5, 22.5 and 27.5°C). Isolate MA2 displayed the lowest growth rate at 27.5°C (0.11 mm day^{-1}), with the maximum growth rate at between 15°C (4.57 mm day^{-1}).

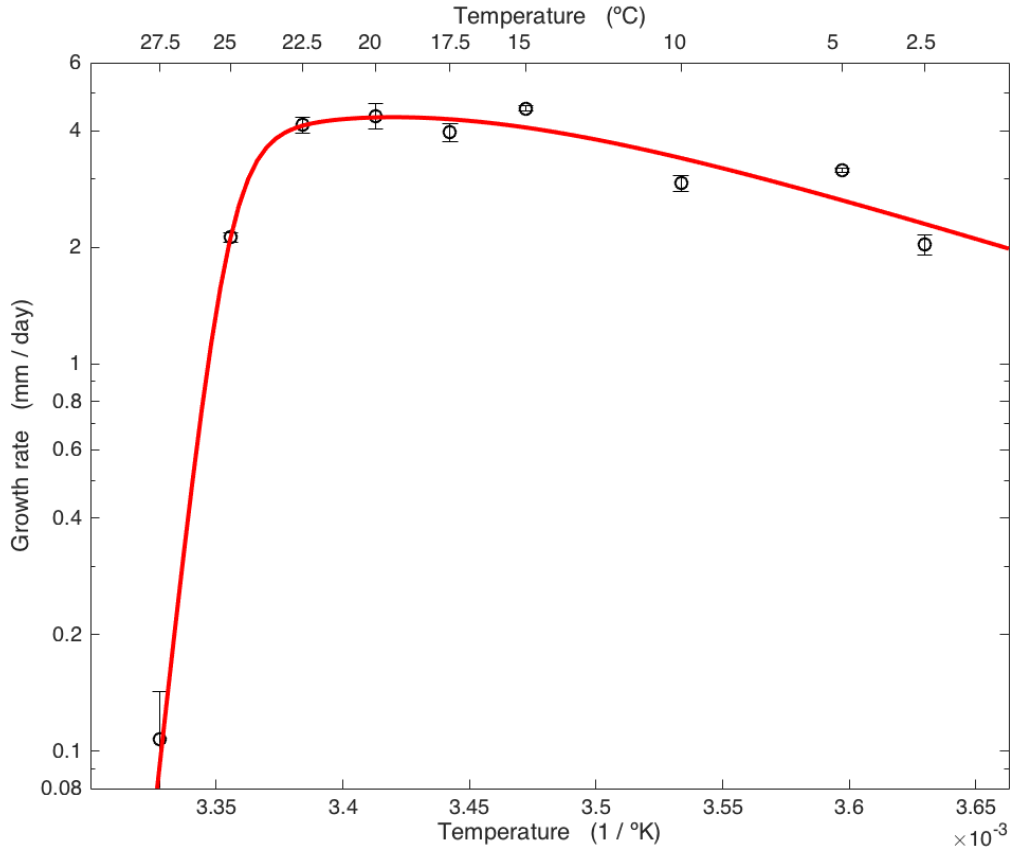


Figure 4.15 – Model predictions for the effect of temperature on the growth rate of *M. acerina* isolate MA2 (Table 4.3) on an Arrhenius plot. Red line represents fitted curve obtained using equation (2) for MA2 grown on PDA, with grey triangles representing mean growth rate of four replicates; error bars show SEM.

Table 4.10 – Summary of parameter estimates for the effect of temperature on the extension rates of colonies of *M. acerina* isolates and *M. acerina* standard isolate MA2. Estimates based on the modified Schoolfield *et al.* (1981) equations (2) and (3), the six parameter and four parameter model, respectively, as described in the text.

	$\rho_{(15°C)}$ mm day^{-1}	$\Delta H_A^{\neq}$ cal mol^{-1}	ΔH_H cal mol^{-1}	$T_{1/2_H}$ °C	ΔH_L cal mol^{-1}	$T_{1/2_L}$ °C
M. acerina isolates	4.1216	9536.2	1.45E+05	22.126		
M. acerina isolate MA2	25.685	-22175	2.56E+05	24.705	-30824	24.194

The parameter estimates for the model fitting (Table 4.10) vary significantly between the four parameter model (equation (3)) fitted to all the *M. acerina* isolates and the six parameter model (equation (2)) fitted to the standard *M. acerina* isolate, MA2. As an example, $\rho_{(15°C)}$, the estimated rate of growth at 15°C (assuming no enzyme inactivation), is 4.12 mm day^{-1} for all the *M. acerina* isolates and 25.69 mm day^{-1} estimated for *M. acerina* isolate MA2. However, in contrast to the model fitting for *C. destructans*, the parameter estimates when fitting all *M. acerina* isolates are biologically more reasonable that the model fitted to *M. acerina* isolate MA2 only. This can be attributed to two main reasons. First, between 10°C and 25°C there is little variability between isolates (see Table A.7 in the appendix) at each temperature so a single model can be fitted to all *M. acerina* isolates. Second, when measuring the additional temperatures, the growth rate at 27.5 (0.11 mm day^{-1}) is significantly lower than that at 2.5 (2.04 mm day^{-1}), the lack of a drop in growth rate at low temperatures made the fitting of the six parameter model challenging, and resulted in inaccurate parameter estimates.

4.3.9 Effect of UV light on *M. acerina* spore production

The number of spores produced by *M. acerina* isolates was assessed at 20°C, under both UV conditions and darkness. Evenhuis et al (1997) found UV light to stimulate sporulation, and represent conditions similar to those observed in summer. This experiment therefore simulates comparative spore number during summer and winter conditions. The general trend of the data suggested an increase in spore production in all *M. acerina* isolates under UV conditions (Fig 4.16). Spore numbers produced under UV conditions ranged from 266000 (MA7) to 903000 (MA2), and from 35250 (MA1) to 317000 (MA5) in dark conditions. Isolates MA2 and MA5 display higher spore counts under both lighting conditions compared to other isolates, whilst MA1 and MA3 had the lowest spore counts under dark conditions and MA4 and MA7 had the lowest spore counts under UV light. Overall, MA2 and MA5 displayed high spore counts under both lighting conditions, and MA3 and MA4 displayed low spore counts under both lighting conditions.

The ANOVA showed a significant effect of *M. acerina* isolate ($p<0.001$) on spore production, and light conditions to have a significant effect on spore production ($p<0.05$). The posthoc 'Tukey' analysis identified significant differences between *M. acerina* isolates under the two lighting conditions. During dark conditions MA4 (median spore producer) was significantly different ($p<0.01$) to all other isolates, with the remaining six isolates displaying a significant difference ($p<0.05$) to at least four other isolates. Under UV conditions, *M. acerina* isolate MA1 (high spore producer) was significantly different ($p<0.05$) to all other isolates, with the remaining six isolates displaying a significant difference ($p<0.05$) to at lest three other isolates. The high number of significant interaction between isolates may be due to the small error (SEM) for all isolates. Full results of the 'Tukey' analysis can be found in the appendix (Table A.8 in the appendix).

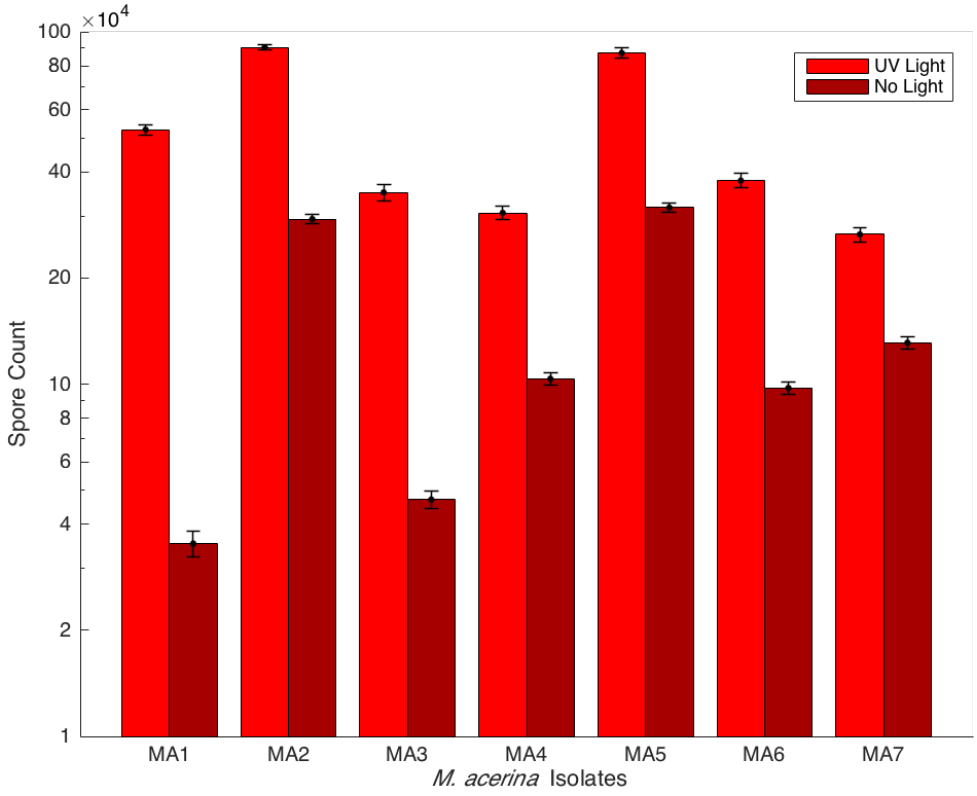


Figure 4.16 – Spore count at 20°C for *M. acerina* isolates (Table 4.3) grown on PDA for 10 days under dark and UV light conditions. Bars represent mean spore count from four replicates, error bars show SEM.

4.3.10 *M. acerina* molecular characterisation

Following confirmation of *M. acerina* species identity through *ITS* sequencing and BLAST analysis sequence data for the housekeeping genes *ITS*, *EF-1α*, *Rpb-II* and *TUB2* were assembled and aligned for all *M. acerina* isolates.

The maximum likelihood and neighbour joining analyses for the *ITS* locus separated *M. acerina* isolates into two and three clades respectively (I and II) (Fig 4.17 and 4.18), with the only difference between the two analyses being the placing of an *M. acerina* isolate from GenBank (AY266155.1). The larger clade for both analyses contains 10 isolates, and the smaller containing three isolates, with the former subdivided into two genetic clusters. The larger clade (I) on both analyses contains *M. acerina*, *M. cantuariensis* and *M. delegatensis* isolated from parsnip, eucalyptus, ginseng, peony and hop across UK, The Netherlands, Chile, China, Austria and Ethiopia. This suggests sequences within this clade are not related to host species or geographic location. The three isolates comprising the other clade (MA3, MA4 and MA6) were all isolated from parsnip grown in Lincolnshire. This could indicate some species clustering based on both host and location; however, isolates MA1, MA2, MA5 and MA7 in the larger clade were also all isolated off parsnip from Lincolnshire, further suggesting that clustering is not related to host and location. Bootstrap values on the maximum likelihood analysis ranged from 3% to 100%, with the majority above 80%. The neighbour joining analysis bootstrap values were all above 60%, with the majority above 97%. These values indicated the majority of branches in the two analyses were well supported.

Concatenated alignments of the housekeeping genes *ITS*, *EF-1α*, *Rpb-II* and *TUB2* were analysed using both maximum likelihood and neighbour joining analyses (Fig 4.19 and Fig 4.20). Both phylogenies are identical and divide the *M. acerina* isolates into two clades, of four and three isolates respectively. None of the isolates appear to be related by host or geographical location, as all isolates are from parsnip grown in Lincolnshire, yet appear in two different clades. The

bootstrap values for both the maximum likelihood and neighbour joining analysis range from 72% to 100%, with the majority of branches showing values of 100%, indicating good support for branches.

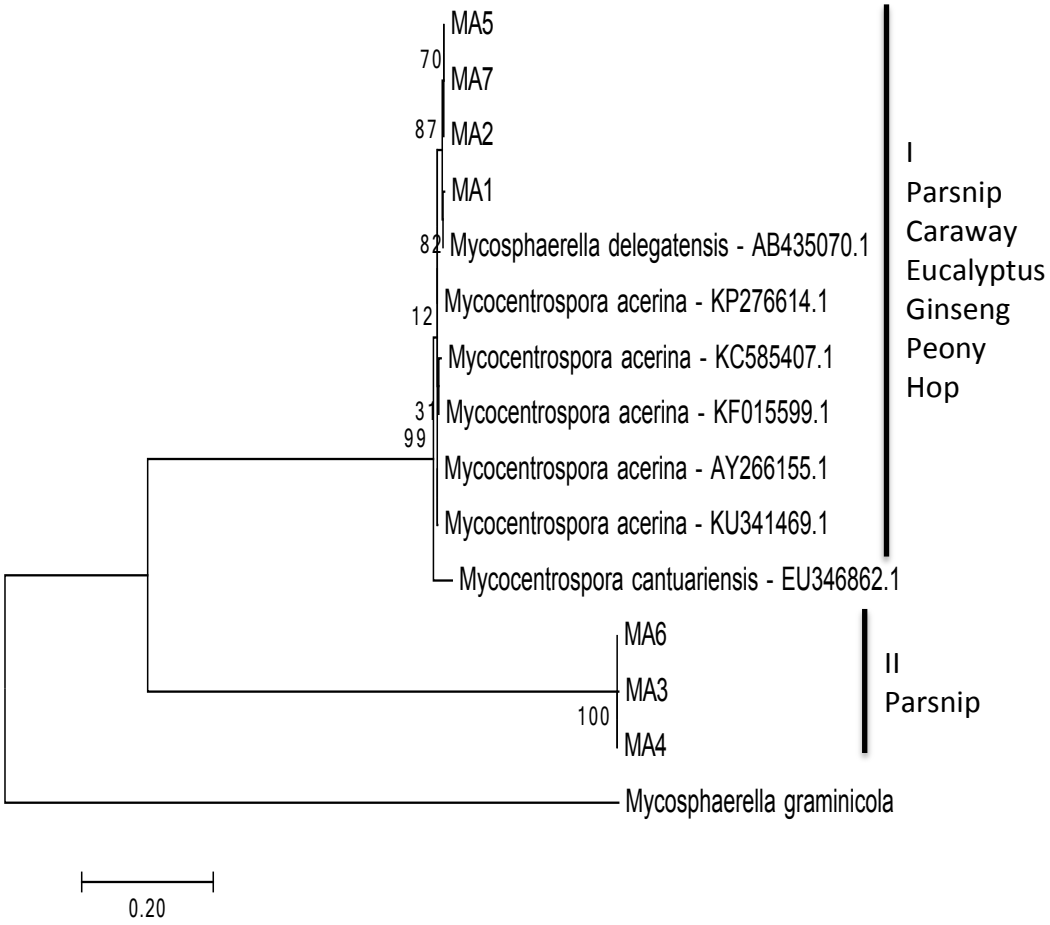


Figure 4.17 – Maximum likelihood phylogenetic tree for *M. acerina* isolates based on an alignment of the *internal transcribed spacer (ITS)*. Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.20 substitutions per site. The tree is rooted through *Mycosphaerella graminicola* (Goodwin *et al.*, 2011), with reference *M. acerina*, *M. cantuarlensis* and *M. delegatensis* isolates (Table 4.6).

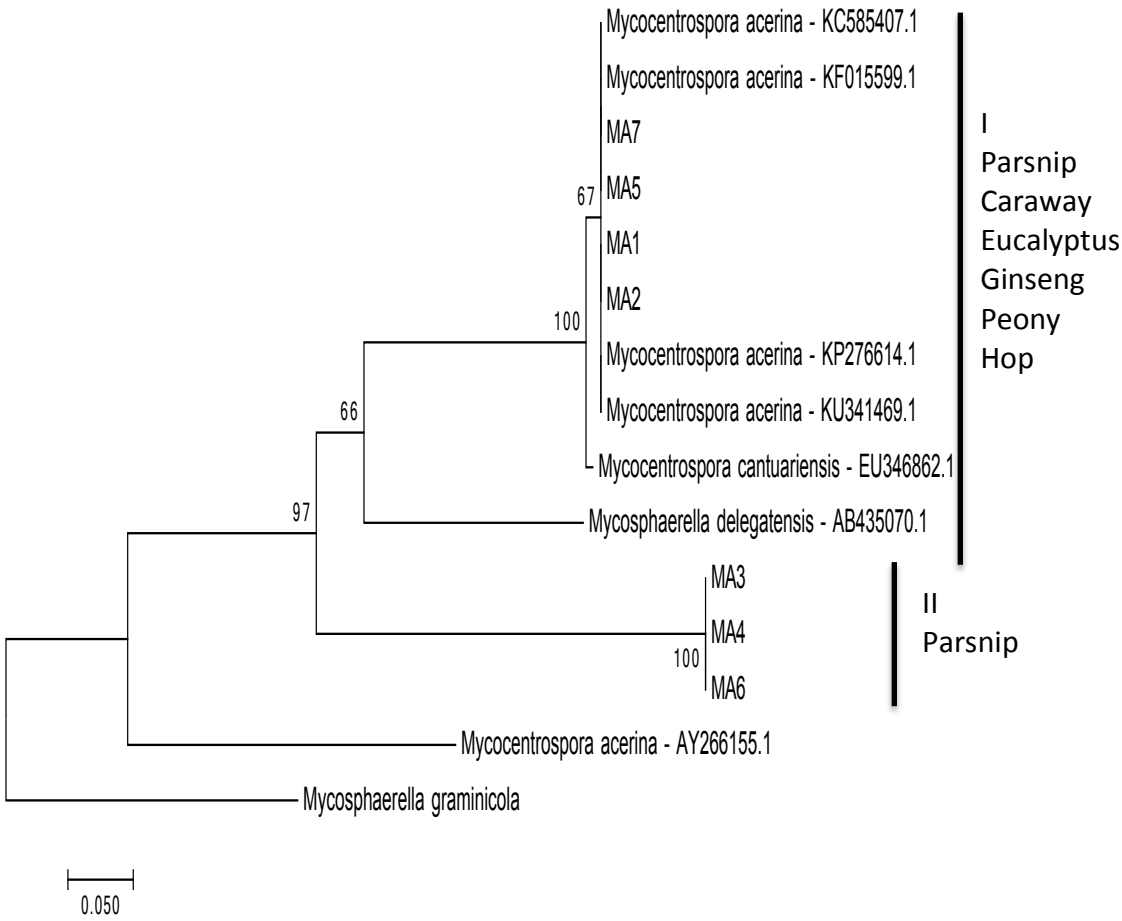


Figure 4.18 – Neighbour Joining phylogenetic tree for *M. acerina* isolates based on an alignment of the *internal transcribed spacer (ITS)*. Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site. The tree is rooted through *Mycosphaerella graminicola* (Goodwin *et al.*, 2011), with a reference *M. acerina*, *M. cantuarlensis* and *M. delegatensis* isolates (Table 4.6).

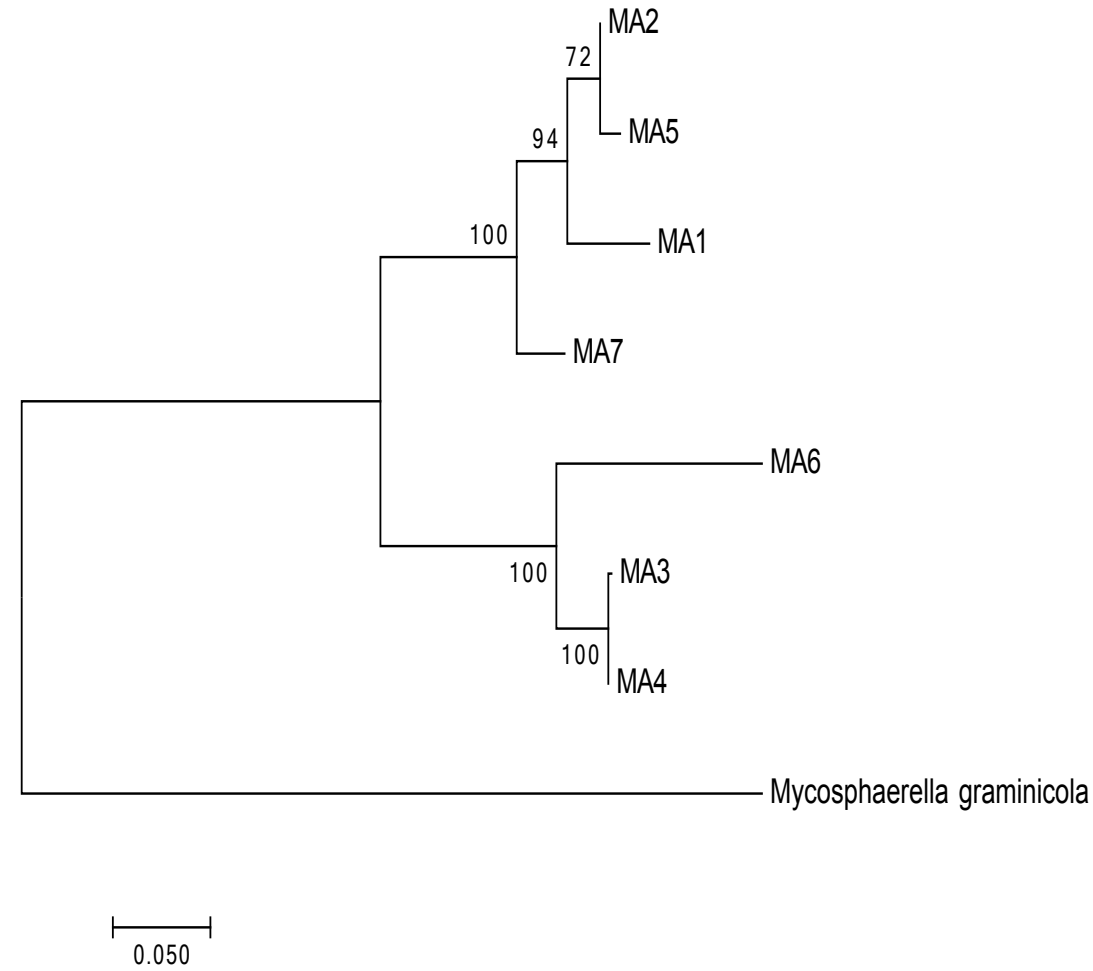


Figure 4.19 – Maximum Likelihhood phylogenetic tree for *M. acerina* isolates based on an alignment of the internal transcribed spacer (*ITS*), RNA polymerase II (*rpb-II*), translation elongation factor (*EF-1α*) and Beta-tubulin (*TUB2*). Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site. The tree is rooted through *Mycosphaerella graminicola* (Goodwin *et al.*, 2011).

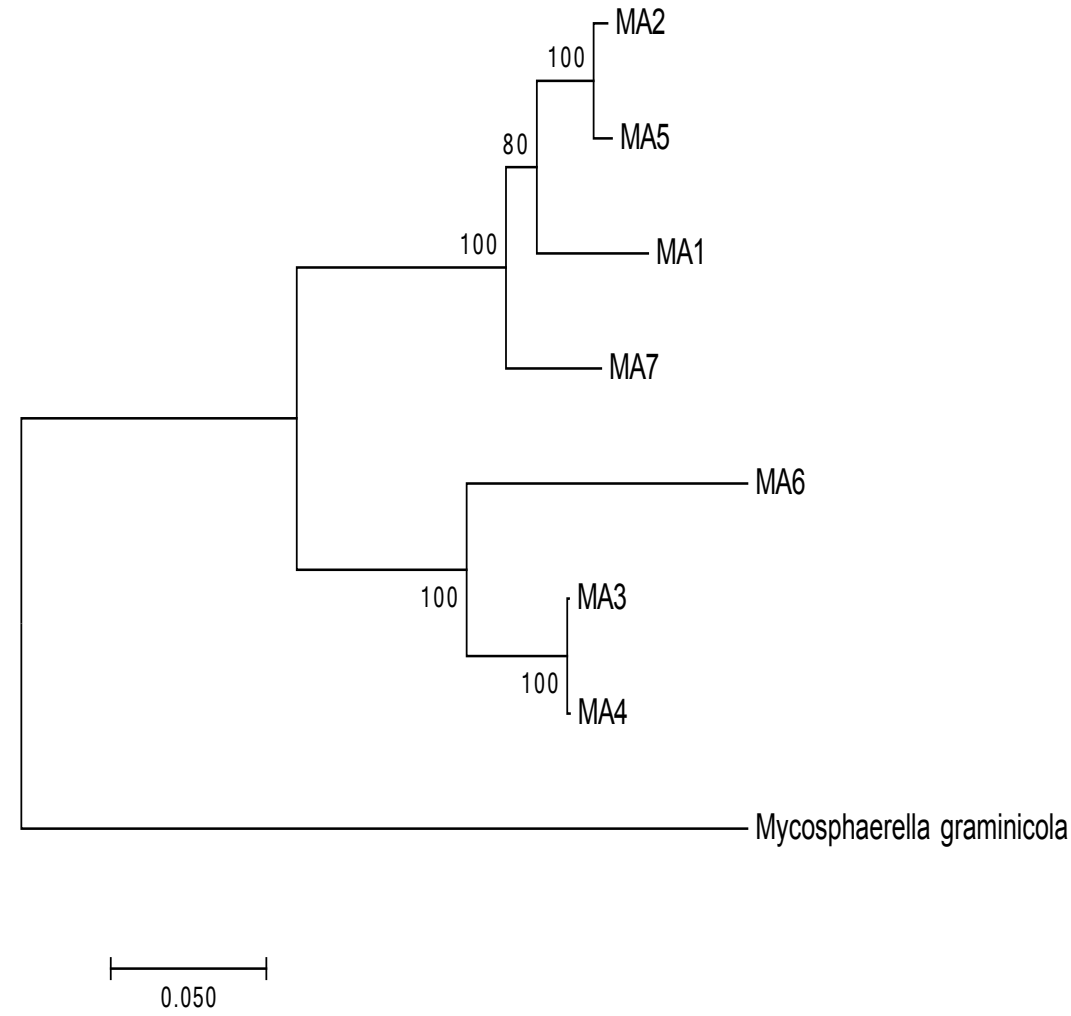


Figure 4.20 – Neighbour Joining phylogenetic tree for *M. acerina* isolates based on an alignment of the internal transcribed spacer (*ITS*), RNA polymerase II (*rpb-II*), translation elongation factor (*EF-1α*) and Beta-tubulin (*TUB2*). Numbers represent bootstrap values from 1,000 replicates. Scale bar indicates 0.050 substitutions per site. The tree is rooted through *Mycosphaerella graminicola* (Goodwin *et al.*, 2011).

4.4 Discussion

To date, there have been no studies concerning the diversity and characteristics of *C. destructans* and *M. acerina* isolated from parsnip since the work of Channon (1965, 1981). Defining the biological characteristics of these two pathogens is important in understanding their epidemiology, assessing disease risk and developing control methods.

In the *C. destructans* root pathogenicity tests, all isolates led to the development of lesions on parsnip roots but there was a range of virulence as demonstrated by significant differences in lesion size. However, no defined groups of isolates were identified as more or less pathogenic than the rest, as a continuum of variation was observed. These results are in line with the work of Channon (1981), where a range of pathogenicity on parsnip roots was also detected between 8 *C. destructans* isolates. However, Channon (1981) detected four weakly pathogenic isolates, causing lesion areas of 1-3 cm^2 and four highly pathogenic isolates, resulting in lesion areas of 9-14 cm^2. The lesion areas observed here ranged from 0.5-2.0 cm^2, exhibiting values similar to the lesions of the weakly pathogenic isolates described by Channon (1981). In the *C. destructans* seedling assay, all isolates led to seedling death or brown root symptoms. Statistically significant differences were again detected between the isolates, with five isolates being identified as significantly more pathogenic than the others. As no previous work has investigated the effect of *C. destructans* isolates on parsnip seedlings, the most comparable work is that of *C. destructans* on grapevine. Úrbez-Torres (2014) found a range of pathogenicity between *Cylindrocarpon* spp. on grapevine: after inoculating grapevine canes with mycelial plugs, he detected a range of vascular discolouration of 5.6-11 mm after 31 days at room temperature, with a substantial reduction in vigour and reduced shoot growth also documented in some cases (Úrbez-Torres, 2014). In contrast to the basal stem inoculation method of 1 x 10^5 used in the current experiment, Rego *et al.* (2001) carried out grapevine root dipping at 10^8 suspensions for 30

minutes. This resulted in root lesions and vascular discolouration, displaying a great variety in virulence between isolates (70-100%), but much less than that observed in this study (10-85%).

The current study represents the first comparison between parsnip seedling and root pathogenicity assays for *C. destructans* isolates from parsnip hosts. However, there was little consistency between the pathogenicity of isolates in the two assays, with only 3 isolates scoring low (CD18, CD23 and CD24) and only 2 isolates being highly pathogenic (CD21 and CD28) in both assays. Numerous isolates displayed contrasting levels of pathogenicity, with CD19 being weakly pathogenic on root and highly pathogenic on seedlings, and CD1, CD10, CD30 and CD31 showing the opposite trend. There is no clear reason as to the lack of correlation between isolates; however, as the two experiments were carried out at different temperatures (12 °C for root and 20 °C for seedlings), it could be hypothesised that different isolates are more pathogenic at different temperatures.

The experiments examining the effect of temperature on mycelial growth rate showed that maximum growth rate across all *C. destructans* isolates was around 20°C, with the lowest rate of growth at 5°C. Significant differences between isolates were mostly observed at the more extreme temperatures tested of 5 and 25°C, potentially suggesting adaptation. The standard *C. destructans* isolate CD28 was selected to test additional temperatures, to fully investigate the parameters of the model of Schoolfield *et al.* (1981) that control enzyme inactivation due to both high and low temperatures. The lowest rate of growth for *C. destructans* isolate CD28 was observed at 2.5°C for both CDA and PDA, with the maximum growth rate at 22.5°C on both PDA and CDA. These results are in agreement with those of Alaniz *et al.* (2007), who examined the growth rate of *C. microdidymum* and *C. liriodendra* on PDA at temperatures 5-35°C and observed maximum growth at 20-25°C. Alaniz *et al.* (2007) also reported slow growth at 5°C but did not examine lower temperatures. Furthermore, it is

generally accepted that the majority of saprophytic fungi do not grow below 5°C (Ayerst, 1969). In contrast, for *C. destructans* CD28, growth was observed at a temperature of 2.5°C. This is the first time *C. destructans* isolates from parsnip have been found to grow at such low temperatures. Therefore, whilst production of chlamydospores is known to be a mechanism for pathogen survival throughout winter, this research suggests *C. destructans* isolates are also capable of mycelial growth at temperatures typical of UK winter. In addition, as previously mentioned, few fungi are documented as growing at temperatures below 5°C: it is therefore probable that the increase in *C. destructans* incidence over winter may also be due, in part, to reduced activity of, and hence competition with, other saprophytic fungi. However, further research is needed to confirm the lowest temperature at which *C. destructans* mycelial growth can occur.

In assessing the effect of temperature on *C. destructans* chlamydospore production, the general trend in the data suggested that spore density was generally consistent across all temperatures examined. This was surprising as, given chlamydospores are long-lived structures (Taylor, 1964) capable of surviving over winter (McPherson, 2013), it could hypothesised that isolates might produce an increased number of chlamydospores at lower temperature. However, this was not the case. Whilst spore production remained constant across temperatures, differences between isolates became more apparent with increasing temperature. Alaniz *et al.* (2007) examined conidia sporulation in 82 *Cylindrocarpon* spp. isolates at 24°C for 7 days and recorded huge variations in conidia production, with values of 13.0-2.2 x 10^6.conidia ml^{-1}. Whilst less pronounced, wide variations in spore production between isolates was also observed at this temperature in the current research, with values in the range 0.1-453.52 chlamydospores mm^{-2}, suggesting that some isolates are better adapted than others to high temperatures. The understanding of how temperature affects *C. destructans* spore production is potentially important, as this information could be used to more accurately target specific control

methods. However, despite chlamydospores being implicated in causing high canker incidence over winter (MacPherson, 2013), chlamydospore production does not appear to be affected by temperature. Therefore, no high risk season can be identified, perhaps suggesting routine fungicide application may be more effective than specific targeted use.

Both the maximum likelihood and neighbour joining phylogenetic analysis based on the *ITS* locus divided isolates into three clades. Ten of the *C. destructans* isolates are grouped in a single clade, together with isolates from the *C. destructans* anamorph, *N. radicicola*, from grapevine and pine. The remaining six isolates (CD5, CD10, CD20, CD22, CD30, CD31) were distributed across the other two clades, where they appear more closely related to *C. theobromicola*, *C. obtuisisporum*, and *C. cylindroides* than *C. destructans*. Within the root pathogenicity assay, these six isolates were among the most pathogenic, whilst in the leaf pathogenicity assay, they scored amongst the least pathogenic. In particular, two of the isolates, CD30 and CD31, which were the most closely related to *C. theobromicola* and *C. obtusisporum*, also displayed consistently higher growth rates compared to the other *C. destructans* isolates. The other four isolates CD5, CD10, CD20 and CD22 were grouped in the same clade as *C. cylindroides*, which was originally isolated from fir. It could be hypothesised that these *C. destructans* isolates may also have originated from fir, based on the knowledge that the parsnips from which they were isolated were grown in fields adjacent to extensive pine forests (personal communication). As shown by Seifert *et al.* (2003), *C. destructans* isolates from conifer were also found to cause severe footrot in ginseng seedlings, proving a lack of host specificity. Seifert *et al.* (2003) also detected a high level of divergence in the *ITS* locus of *N. radicicola*, with isolates from a range of hosts being present in all clades, and further observed some *C. destructans* isolates from black spruce hosts to be more closely related to *C. cylindroides* than other *N. radicicola* isolates. This is in line with the results obtained here, where *N. radicicola* from the same host is present in all clades, and the four *C. destructans* isolates described above appear most

closely related to *C. cylindroides*. Given *C. cylindroides* has been isolated from fir hosts, this further confirms the idea that those four isolates in the separate clade may be more closely related to *C. cylindroides*. Similarly, in both the maximum likelihood and neighbour joining analysis of the multigene phylogeny based on the *ITS*, *EF-1* α, *Rpb-II* and *TUB2* loci, the *C. destructans* isolates again separate into two clades, with the same *C. destructans* isolates CD5, CD10, CD20 and CD22, and CD30 and CD31 grouped separately as for the *ITS* phylogeny alone. This confirms the work of Cabral *et al.* (2012), who observed multiple species present across all clades and hence concluded that the *Cylindrocapon*-like species are part of a species complex.

The root pathogenicity assay examining differences between *M. acerina* isolates identified a range of pathogenicity, but no significant differences were observed between isolates. This could be due in part to the small sample size, and reduced sampling locations, of the *M. acerina* isolates. The *M. acerina* seedling pathogenicity assay further identified little variation in pathogenicity, with each isolate causing similar levels of infection. Therefore, no individual *M. acerina* isolates could be clearly identified as being more or less pathogenic. In contrast, Channon (1965) observed a difference in the pathogenicity of *M. acerina* isolates from parsnip hosts, on re-inoculated parsnip roots as well as carrot and celery. He also noted that lesions were more extensive on parsnip and celery, compared to the dry, shallow lesions on carrot. Further pathogenicity assays should focus on infecting different hosts with the isolates obtained here to confirm, as the work of Channon (1965) suggested, that pathogenicity may vary between different hosts.

The effect of temperature on the growth rate of *M. acerina* isolates identified the maximum growth rate to be at 20°C, whilst the minimum growth rate was observed at 25°C (though with large variations between isolates). This confirms the results of Channon (1965), who identified maximum mycelial growth between 18.5 and 21.5°C, with little growth at 29°C. Although growth at such

high temperatures was not tested here, it could be extrapolated from the model fitting to be occurring at a very low rate, if at all. The presence of UV light was found to consistently increase spore production across all *M. acerina* isolates. Although significant differences were detected among the isolates, results did not correlate with those of any other assays, meaning isolates displaying higher growth rates than others were not necessarily more pathogenic on roots or seedlings. Although not observed in parsnips, Hermansen (1992) identified *M. acerina* as the causal agent of foliar symptoms on a range of wild hosts, including umbellifers, whilst Evenhuis (1995) found that *M. acerina* could be transmitted through infected caraway seed. This suggests *M. acerina* may be a seed borne pathogen, with an aerial dispersal method. As the spore production experiments found a higher number of spores produced under UV light, this suggests an increased spore production during the summer. Therefore, it could be hypothesised that the increased conidia production during the spring and autumn (Arsvoll, 1969) results in aerial transmission throughout the summer when foliage is present, whilst the abundance of chlamydospores produced in senescing tissue throughout autumn (Day *et al.* 1972; Davies, 1977) results in necrotic tissue falling into the soil leading to a build up of inoculum. As conidia have been found to become chlamydosporic (Wall & Lewis, 1978), the increased production of conidia in autumn is also likely to lead to increased chlaymdospores in the soil. The viability of chlamydospores within the soil declines very slowly, with Wall & Lewis (1980) noting that after two years the fungus grew from 99% of spore samples buried at a depth of 5 mm, and 97% of spore samples buried at a depth of 20 mm, with no indication of any decline in pathogenicity (Wall & Lewis, 1980). This means crops sown the following season could be infected by the chlamydospore soil inoculum from previous years and therefore crop rotation is unlikely to appreciably reduce chlamydospore levels in the soil. However, further work to develop spore trapping and q-PCR methods would enable more accurate estimates of spores in the air and soil. This in turn could be used to further advise control methods.

The molecular characterisation of *M. acerina* isolates have been described through a single gene phylogeny based upon the *ITS* region, and a multigene phylogeny based upon the *ITS*, *EF-1* α, *Rpb-II* and *TUB2* loci. Both the maximum likelihood and neighbour joining *ITS* analysis split the *M. acerina* isolates into two clades, one containing three isolates and the other containing the four remaining *M. acerina* isolates from parsnip together with all other *M. acerina* sequences from GenBank, isolated from a range of hosts. This analysis failed to distinguish between different *Mycocentrospora* species from both parsnip and other hosts. This could be due, in part, to the small sample size, but it is more suggestive of a species complex. Given the *ITS* locus, the primary fungal barcode for species differentiation, was not able to distinguish between species, it could suggested further multigene analyses may not be necessarily helpful. One feature of interest is the separation of *M. acerina* isolates MA3, MA4 and MA6 from parsnip into the same clade. This result does not correlate to any biological characteristics observed in the other assays, or to sampling location. However, it could be an indication of these isolates being adapted preferentially to parsnips. Both the maximum likelihood and neighbour joining multigene analyses allowed a finer resolution in isolate differentiation, but due to limited sequence data on GenBank further investigation would be required to fully resolve this species.

In conclusion, this work developed novel methods for assessing the pathogenicity of *C. destructans* and *M. acerina* on parsnip seedlings, and represents the first analysis where isolates from parsnip have been directly compared. It is also the first comprehensive analysis of the effect of temperature on mycelial growth and spore production, therefore providing a more robust basis for investigating intra species diversity. This study was also the first multilocus sequence comparison for both *C. destructans* and *M. acerina* isolates from parsnip. Results suggest both these pathogens may belong to species complexes, where differentiation between species in difficult.

Previous work has found both pathogens to produce long-lived chlamydospores thought to be responsible for high canker incidence during winter, whilst a number of wild species have been identified as hosts, and potential sources, of inoculum. The results obtained here additionally confirm both *C. destructans* and *M. acerina* are able to grow at low temperatures, with minimal differences between the pathogenicity of isolates on parsnip roots and seedlings. This information could be used to better inform control approaches and breeding programmes to provide more effective control against these two pathogens.

5. Characterising resistance in parsnip to *I. pastinacae*, *M. acerina* and *C. destructans*

5.1 Introduction

Parsnips are grown on approximately 3000 ha across the UK, producing a total of 93,000 tonnes with an annual economic value of over £31M (Kantar Worldpanel, 2015; Crown Copyright, 2014; Defra, 2014). Visual appearance is a major trait for their marketability and the greatest losses and economic damage to parsnip crops is due to fungal pathogens causing root canker diseases (Gladders, 1997). Whilst there are no official figures, the industry predicts an average 20% annual loss, and up to 50 – 80% losses in overwintered crops due to root canker diseases (McPherson, 2013; personal communication with Elsoms Seeds Ltd; 2016).

There are few control methods considered robust enough to prevent root canker diseases. Current cultural practices such as good crop rotation, early and widely spaced sowings and covering of exposed roots with soil are frequently practiced but often ineffective due to environmental conditions with cold, damp seasons and high summer rainfall leading to increased canker incidence (Channon, 1964, Wilkinson, 1952; Brown *et al.*, 1964). No specific fungicides are currently available for use against root cankers, with non-specific fungicides such as Amistar (200 g / L azoxystrobin), Topsin (70% w/w thiophanate-methly) showing promise in previous early trials (Gladders, 1997). No further research was carried out into the effect of these fungicides against root canker pathogens. However, Amistar and Reflect (125 g / L isopyrazam) are both regularly used by growers to offer all round disease control to both foliar and root pathogens (personal communication). As Itersonilia is a seed-borne pathogen, seed treatment options have been explored with Smith (1966) noting that steam treatment (45.5°C for

30 minutes) successfully removed Itersonilia without damaging the seed or reducing germination. The long-lived survival structures, chlamydospores, produced by all three of the main canker pathogens, *I. pastinacae*, *M. acerina* and *C. destructans,* are most problematic in controlling root diseases. These structures can survive in the soil at cold temperatures for prolonged periods, making them difficult to eradicate (Evenhuis, 1995).

Breeding for resistance to parsnip canker has been implemented among commercial breeding companies to enable long-term, robust resistance to parsnip root diseases. However, in previous trials to breed for resistance, there have been concerns over the loss of other marketable traits. For example, the Avonresister breeding programme started in 1953 and the variety was bred for canker resistance using parent roots of medium length with intrinsic resistance to various forms of canker. This line was successful in showing generalised resistance to canker but, due to its small root size, other varieties outyielded it by 88%, (Channon *et al.*, 1970). Parsnips, like carrots and onions, are outbreeding crops and so varieties are highly heterogenous, making the task of breeding uniform lines more difficult. However, vigorous F_1 hybrids can be developed by taking advantage of the heterosis resulting from crossing suitable lines to introduce favourable traits such as increased yield. In addition to high heterogeneity, parsnips also suffer from inbreeding depression as a result of repeated selfing, which produces progeny with reduced fitness. Therefore, backcrossing is frequently used, where an F_1 hybrid is crossed with a parent to produce progeny displaying favourable traits closer to that of the parent. The process results in uniform progeny with high vigour. This process of traditional breeding can take up to 20 years to produce a new variety, but through the application of marker assisted breeding this time can be significantly reduced (personal communication; Elsoms Seeds Ltd; Collard *et al.*, 2005).

A number of agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by multiple genes and are known as

quantitative traits, with the regions of the genome associated with a particular trait known as quantitative trait loci (QTLs). Identification of QTLs through traditional phenotyping alone is not possible; instead, they are identified through a combination of phenotyping and DNA markers via QTL mapping (Collard et al., 2005). Single nucleotide polymorphisms (SNPs) and the availability of high-throughput genotyping platforms have accelerated the generation of linkage maps and the routine use of marker-assisted breeding (Collard & Mackill, 2008). SNP discovery does not always guarantee a valid marker, so validation methods are required. Of the available methods, LGC Genomics' (formerly KBiosciences) Competitive Allele Specific PCR (KASPar) is considered one of the most cost-effective, and due to the flexibility of the assay, data can be obtained very quickly (Kumpatla *et al.*, 2012). This type of marker is now also widely used by the breeding industry.

KASP (Kompetitive Allele Specific PCR) is a uniplex SNP genotyping platform that offers cost-effective and scalable flexibility in applications that require small to moderate numbers of markers, such as quantitative trail loci (QTL) mapping in bi-parental crosses, marker assisted selection and marker assisted backcrossing (Semagn *et al*., 2013). KASP is a homogeneous, flurescence-based genotyping technology. It is based on allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation (Kumpatala *et al*., 2012). The chemistry of the assays involves one common reverse primer and two competitive allele specific tailed forward primers, then to determine alleles at a specific locus, the system relies on the apttern of competitive allele specific PCR (Lateef, 2015). This involves competitive allele-specific PCR for a given SNP, followed by SNP detection through FRET (Mir *et al*., 2013). In KASPar assays there is no sequencing requirement to identify SNPs, instead SNP flanking sequences already known from other genotyping assays (such as Illumina), can be used for primer design for the KASPar assays (Gupta *et al*., 2008). Whilst KASPar technology is still considered relatively new to the market, it is being widely used for a large number of commercial species such as maize and wheat,

and offers an accuracy, speed and cost-effectiveness not currently available in other marker technologies. It is for these reason this type of marker is widely used by the breeding industry, and the reason for its use in this work.

There is no current literature exploring large-scale phenotyping or the genetic basis for parsnip root canker resistance. Therefore the specific aims of this work were:

- To determine levels of resistance within a parsnip parent line population through the development of novel parsnip root and seedling assays;
- To determine levels of resistance within a parsnip breeding line and $F_{2:3}$ genotyping population;
- To generate a linkage map based on the P20 x P22 F_2 mapping population and identify QTLs conferring disease resistance.

5.2 Materials & Methods

5.2.1 Parent line root assay to screen for resistance to *I. pastinacae, M. acerina and C. destructans*

Experiments were set up to evaluate disease resistance on roots from a parsnip parent line population from Elsoms Seeds Ltd. Ten parent lines (Table 5.1) were grown from seed in 2:1 mix of Levington F_2 compost and sharp sand (Everris, UK) in 10 L pots, with seven plants per pot. All pots were placed into a glasshouse compartment at 20°C, to mature and allow roots to develop. After 6 months the parsnip roots were harvested, washed with tap water, surface sterilized with 70% ethanol (v/v) and air-dried. Each root was checked for any disease symptoms before the widest part of the root was inoculated with a 5 mm agar plug of either *I. pastinacae* isolate IP10, *M. acerina* isolate MA2 or *C. destructans* isolate CD28 from a seven-day-old actively growing colony (as described in Section 3.2.1, Chapter 4, Section 4.2.1 and Section 4.2.7). The parsnips were then incubated at 12°C on damp tissue in sealed 3 L plastic boxes with moist conditions maintained to encourage disease development by misting the roots with RO water once a week.

Table 5.1 – List of ten parsnip parent lines from Elsoms Seeds Ltd.

Number	Parsnip Line
1	Panache
2	P9
3	P11
4	P14
5	P15
6	P20
7	P22
8	P23
9	P24
10	P25

One experimental replicate consisted of six roots for each parent line-pathogen combination in two plastic boxes, and three repeat experiments were carried

out. Photographs of symptoms were taken weekly from three weeks post inoculation and lesion area measured using ImageJ (https://imagej.nih.gov/ij/).

5.2.2 Parent line seedling assay to screen for *I. pastinacae* resistance

Experiments were set up to assess the resistance of the same parsnip parent lines as used in the root resistance assays to *I. pastinacae* isolate IP10 (Section 5.2.1, Table 5.1). This isolate was first subcultured onto 2% malt agar (MA) Petri dishes and incubated at 20°C for ten days. Three agar blocks taken from the leading edge of the culture were placed into a Petri dish of 20 ml Malt Extract Broth (Oxoid) and incubated for ten days at 20°C. Agar plugs were removed and mycelium washed in sterile RO water before being weighed. Three times the weight of the mycelium of sterile RO water was added to the mycelium and homogenised (MSE, England). A total of 1,000 seeds per 100 ml were soaked in the macerated mycelia for four hours, after which the excess fluid was drained off and the seed left to dry overnight in a laminar flow cabinet. A seed test was carried out to confirm all seeds were coated in inoculum. Thirty-five seeds were sown to a depth of 0.5 cm in a clear plastic box containing 250 g sieved sharp sand, and 20 ml sterile RO water added to each box before the lid was sealed. Boxes were placed in a controlled environment room at 20°C and a 16-hour photoperiod. Seedlings were assessed for germination and mortality twice weekly for a total of forty days. After this time seedlings were harvested and scored for the presence of brown root symptoms. As *I. pastinacae* is a seed-borne pathogen, background levels of the pathogen were also present on the non-inoculated control seed. Therefore, an equal number of replicates for the control group as for the inoculated group were carried out. Four boxes were sown for each control and inoculated parent line seed, with a total of four replicate experiments carried out.

5.2.3 Parent line seedling assay to screen for *M. acerina* resistance

Seedling assays were set up to assess the resistance of the same parsnip parent lines as used in the root resistance assays (Section 5.2.1, Table 5.1) to *M. acerina* isolate MA2. Cornmeal was added to sieved sharp sand at 100 g L^{-1} and approximately 250 ml of the mixture autoclaved in 500 ml conical flasks. These flasks were inoculated with five 5 mm PDA discs from an actively growing *M. acerina* isolate MA2 colony, grown on PDA for ten days at 20°C. Flasks were incubated at 20°C in darkness for twelve weeks with regular mixing, after which the number of colony forming units (CFU) per gram of inoculum was quantified by plating out serial dilutions. The inoculum was mixed with fresh sharp sand to give final concentration of 1 x 10^4 CFU g^{-1}. Thirty-five seeds were sown to a depth of 0.5 cm in a clear plastic box containing 250 g inoculum. The seeds were covered and 20 ml sterile RO water added to each box before the lid was sealed. Boxes were placed in a controlled environment room at 20°C and a 16-hour photoperiod. Seedlings were assessed for germination and subsequent mortality twice weekly for a total of forty days. After this time, the seedlings were harvested and scored for the presence of brown root symptoms. As previously mentioned, *I. pastinacae* is a seed-borne pathogen with background levels present on control seed. Therefore any brown root symptoms attributed to *I. pastinacae* in the control group were not counted. Four boxes were sown for each control and inoculated parent line seed, with a total of four replicate experiments carried out.

5.2.4 Parent line seedling assay to screen for *C. destructans* resistance

Experiments were set up to assess the resistance of the same parsnip parent lines as used in the root resistance assays (Section 5.2.1, Table 5.1) to *C. destructans* isolate CD28 in a seedling assay. Cornmeal was added to sieved sharp sand at 100 g L^{-1} and approximately 250 mL of the mixture autoclaved in 500 ml conical flasks. The flasks were inoculated with 5 mm PDA discs (five discs per flask) from actively growing *C. destructans* colonies grown on PDA for ten

days at 20°C. Flasks were incubated at 20°C in darkness for twelve weeks with regular mixing, after which the number of colony forming units (CFU) per gram of inoculum was quantified by plating out serial dilutions. The inoculum was mixed with fresh sharp sand to give a final concentration of 1×10^5 CFU g^{-1}. Thirty-five seeds were sown to a depth of 0.5 cm in a clear plastic box of 250 g inoculum. The seeds were covered and 20 ml sterile RO water added to each box before the lid was sealed. Boxes were placed in a controlled environment room a 20°C and a 16 hour photoperiod. Seedlings were assessed for germination and any seedling death twice weekly for a total of forty days. After this time, the seedlings were harvested and scored for the presence of brown root symptoms. *I. pastinacae* is a seed-borne pathogen with background levels present on control seed; therefore any brown root symptoms attributed to *I. pastinacae* in the control group were not counted. Four boxes were sown for each control and inoculated parent line seed, with a total of four replicate experiments carried out.

5.2.5 Breeding line root assay to screen for resistance to *I. pastinacae* and *M. acerina*

A parsnip root assay was developed to evaluate disease resistance within a parsnip breeding line population. Based on feedback from Elsoms Seeds Ltd and parsnip growers, the pathogens *I. pastinacae* and *M. acerina* were selected as the focus for the resistance breeding programme and are therefore the only two pathogens examined in the rest of this chapter. At the time of stating these assays the root symptom identification research (Chapter 2) hadn't yet been completed, as such *C. destructans* was not yet confirmed as a primary causal agent of root canker disease and was therefore excluded from these assays. The breeding line population comprises 27 parsnip lines and are referred to here as B01 – B27. The lines were grown from seed in Levington F2 compost (Everris, UK) in 25 L pots, with 14 seed per pot, at Elsoms Seeds Ltd. The pots were placed in a polytunnel compartment to germinate and encourage root growth. After

approximately 6 months, the parsnip roots were harvested, washed with tap water, surface sterilized with 70% ethanol (v/v) and air-dried. Each root was checked for any disease symptoms before the widest part of the root was inoculated with a 5 mm agar plug of either *I. pastinacae* isolate IP10 or *M. acerina* isolate MA2 from a seven-day-old actively growing colony. The parsnips were then incubated at 12°C on damp tissue in sealed 3 L plastic boxes (three roots to a box) and moist conditions maintained to encourage disease development by misting roots with RO water once a week. Six replicate roots per line for each pathogen, and three repeat experiments, were carried out. Photographs of symptoms were taken weekly from two weeks post-inoculation and lesion areas measured using ImageJ (https://imagej.nih.gov/ij/).

5.2.6 Genotyping population root assay to screen for resistance to *I. pastinacae* and *M. acerina*

A parsnip root assay similar to that used to screen the breeding line population in Section 5.2.5 was also used to evaluate disease resistance within a segregating parsnip genotyping population. This population was derived from the parent line cross P20 X P22 (Table 5.1), and comprises $F_{2:3}$ generation plants generated from seed collected from 140 self-fertilised F_2 individuals (Table 5.2). P20 exhibits resistance to both the *I. pastinaceae* and *M. acerina* pathogens, while P22 is susceptible to both pathogens, so the progeny of the cross were expected to segregate for resistance. The genotyping population plants were grown from seed using the same procedure as in Section 5.2.5, with three repeat experiments being carried out per year. Repeat experiments using *I. pastinacae* isolate IP10 were carried out each year for two years, whilst *M. acerina* isolate MA2 experiments were carried out each year for three years (Table 5.2). Photographs of symptoms were taken weekly from three weeks post-inoculation and lesion area measured using ImageJ (https://imagej.nih.gov/ij/).

Table 5.2 – List of 139 *P. sativa* genotyping Lines ($F_{2:3}$) from Elsoms Seeds Ltd, with pathogen inoculation details for root inoculum (*I. pastainacae* or *M. acerina*) and the year; blank cells indicate missing data.

	***I. pastinacae* (IP10)**		***M. acerina* (MA2)**		
Lines	**2013/14**	**2014/15**	**2013/14**	**2014/15**	**2015/16**
G001		Y	Y	Y	Y
G003	Y	Y	Y	Y	
G005	Y	Y	Y	Y	Y
G006	Y	Y	Y	Y	Y
G007	Y		Y	Y	Y
G011	Y	Y	Y	Y	Y
G013	Y	Y	Y	Y	Y
G014	Y	Y	Y	Y	Y
G015	Y	Y	Y	Y	Y
G017	Y	Y	Y	Y	Y
G019	Y	Y	Y	Y	Y
G020	Y	Y	Y	Y	Y
G021	Y	Y	Y	Y	Y
G025	Y	Y	Y	Y	Y
G026	Y	Y	Y	Y	
G027	Y	Y	Y	Y	Y
G028	Y	Y	Y	Y	Y
G030	Y	Y	Y	Y	Y
G031	Y	Y	Y	Y	Y
G032	Y	Y	Y	Y	Y
G034	Y	Y	Y	Y	Y
G035	Y	Y	Y	Y	Y
G036	Y	Y	Y	Y	Y
G038	Y	Y	Y	Y	Y
G039	Y	Y	Y	Y	Y
G046	Y	Y	Y	Y	
G047	Y	Y	Y	Y	Y
G048	Y	Y	Y	Y	Y
G049	Y	Y	Y	Y	Y
G051	Y	Y	Y	Y	Y
G054	Y	Y	Y	Y	Y
G057	Y	Y	Y	Y	Y
G058	Y	Y	Y	Y	Y
G059	Y	Y	Y	Y	Y
G060	Y	Y	Y	Y	Y
G062			Y	Y	Y
G063	Y	Y	Y	Y	
G064	Y	Y	Y	Y	Y
G065	Y	Y	Y	Y	Y

	I. pastinacae (IP10)		*M. acerina* (MA2)		
Lines	**2013/14**	**2014/15**	**2013/14**	**2014/15**	**2015/16**
G066	Y	Y	Y	Y	
G068	Y	Y	Y	Y	Y
G069		Y	Y	Y	Y
G072	Y	Y	Y	Y	Y
G075	Y	Y	Y	Y	Y
G076	Y	Y	Y	Y	Y
G077	Y	Y	Y	Y	Y
G079	Y	Y	Y	Y	Y
G080	Y	Y	Y	Y	Y
G082	Y	Y	Y	Y	Y
G086	Y	Y	Y	Y	Y
G087	Y	Y	Y	Y	Y
G089	Y	Y	Y	Y	
G091	Y	Y	Y	Y	Y
G092	Y	Y	Y	Y	Y
G103	Y	Y	Y	Y	Y
G105	Y	Y	Y	Y	Y
G108	Y	Y	Y	Y	Y
G111	Y	Y	Y	Y	Y
G119	Y	Y	Y	Y	Y
G124	Y	Y	Y	Y	Y
G138	Y	Y	Y	Y	Y
G139	Y	Y	Y	Y	Y
G140	Y	Y	Y	Y	Y
G142	Y	Y	Y	Y	Y
G143	Y	Y	Y	Y	Y
G144	Y	Y	Y	Y	Y
G151	Y	Y	Y	Y	Y
G152	Y	Y	Y	Y	Y
G153	Y	Y	Y	Y	Y
G159	Y	Y	Y	Y	
G183	Y	Y	Y	Y	Y
G184	Y	Y	Y	Y	Y
G190	Y	Y	Y	Y	Y
G192	Y	Y	Y	Y	Y
G196	Y	Y	Y	Y	Y
G197	Y	Y	Y	Y	Y
G199	Y	Y	Y	Y	Y
G201	Y	Y	Y	Y	Y
G203	Y	Y	Y	Y	
G206	Y	Y	Y	Y	Y
G207	Y	Y	Y	Y	Y
G210	Y	Y	Y	Y	Y

	I. pastinacae (IP10)		*M. acerina* (MA2)		
Lines	**2013/14**	**2014/15**	**2013/14**	**2014/15**	**2015/16**
G211	Y	Y	Y	Y	
G212	Y	Y	Y	Y	Y
G215	Y	Y	Y	Y	Y
G216	Y	Y	Y	Y	Y
G217	Y	Y	Y	Y	Y
G218	Y	Y	Y	Y	Y
G219	Y	Y	Y	Y	Y
G220	Y	Y	Y	Y	Y
G221	Y	Y	Y	Y	Y
G222	Y	Y	Y	Y	Y
G223	Y	Y	Y	Y	Y
G225	Y	Y	Y	Y	Y
G227	Y	Y	Y	Y	Y
G230	Y	Y	Y	Y	Y
G233	Y	Y	Y	Y	Y
G234	Y	Y	Y	Y	Y
G236	Y	Y	Y	Y	Y
G237	Y	Y	Y	Y	Y
G239	Y	Y	Y	Y	Y
G240	Y	Y	Y	Y	Y
G241	Y	Y	Y	Y	
G243	Y	Y	Y	Y	Y
G245	Y	Y	Y	Y	Y
G247	Y	Y	Y	Y	Y
G248	Y	Y	Y	Y	Y
G249	Y	Y	Y	Y	Y
G250	Y	Y	Y	Y	Y
G251	Y	Y	Y	Y	Y
G253	Y	Y	Y	Y	Y
G256	Y	Y	Y	Y	Y
G257	Y	Y	Y	Y	Y
G258	Y	Y	Y	Y	Y
G259	Y	Y	Y	Y	Y
G261	Y	Y	Y	Y	
G262	Y	Y	Y	Y	Y
G263	Y	Y	Y	Y	Y
G266	Y	Y	Y	Y	Y
G267	Y	Y	Y	Y	Y
G268	Y	Y	Y	Y	Y
G269	Y		Y	Y	Y
G270	Y	Y	Y	Y	Y
G271	Y	Y	Y	Y	Y
G273	Y	Y	Y	Y	Y

	I. pastinacae (IP10)		*M. acerina* (MA2)		
Lines	**2013/14**	**2014/15**	**2013/14**	**2014/15**	**2015/16**
G274	Y	Y	Y	Y	Y
G277	Y	Y	Y	Y	Y
G278	Y	Y	Y	Y	Y
G279	Y	Y	Y	Y	Y
G280	Y	Y	Y	Y	Y
G281	Y	Y	Y	Y	Y
G282	Y	Y	Y	Y	Y
G286	Y	Y	Y	Y	Y
G287	Y	Y	Y	Y	Y
G288	Y	Y	Y	Y	Y
G289	Y	Y	Y	Y	Y
G291	Y	Y	Y	Y	Y
G292	Y	Y	Y	Y	Y
Total	135	137	139	139	128

5.2.7 Statistical Analyses

Statistical analysis was carried out using the software R (Version 0.98.945, R Development Core Team, 2014). For the screening of parent lines for resistance to *I. pastinacae*, *M. acerina* and *C. destructans* using a detached root assay, the measured lesion area (cm^2) data was log_e-transformed to satisfy the requirements of homogeneity of variance and reduce the influence of residuals. The transformed data was analysed using a one-way ANOVA and the 5% Least significant difference (LSD) values calculated.

The screening of parsnip parent lines for *I. pastinacae*, *M. acerina* and *C. destructans* resistance using a seedling assay was calculated as seedling disease (%), including seedling death or presence of a brown root symptom on harvest. The data was analysed using a GLM and the posthoc 'Tukey' test applied to determine differences between the parsnip parent lines.

For the screening of parsnip breeding lines for resistance to *I. pastinacae* and *M. acerina* using a detached root test. The measured lesion area (cm^2) data was

analysed using a one-way ANOVA and the 5% LSD values calculated. The lesions area data for *I. pastinacae* were $\log_e$-transformed prior to the analysis.

The screening of parsnip genotyping lines for resistance to *I. pastinacae* and *M. acerina* using a detached root test provided phenotype data for QTL analysis. The data for each pathogen for each year was $\log_{10}$-transformed and analysed using a restricted maximum likelihood (REML). From the REML output, adjusted (least square) means were calculated to compensate for imbalances in experimental design. LSD (5%) values were also calculated to directly compare lines.

5.2.8 SNP genotyping and genetic linkage map construction

SNPs were used to genetically characterize the F_2 *P. sativa* P20 x P22 population. Seed of the P20 and P22 parents was sent by Elsoms Seeds Ltd to Genetwister Technologies B.V. (The Netherlands), where SNPs were identified through transcriptome analysis. A total of 433 SNPs were converted to KASPar™ markers and initially used to genotype 212 individual F_2 plants. This data was used to generate a linkage map in JoinMap 4 (Van Ooijen, 2006) where all the markers were located on eleven linkage groups corresponding to the eleven parsnip chromosomes. However, 64 F_2 plants from which a proportion of the phenotyped $F_{2:3}$ roots were derived were not included in this genotyping assay as the F_2 DNA was not available. To address this, three of the phenotyped $F_{2:3}$ roots for each of the sixty-four F_2 parent plants were genotyped. As many of the 433 markers mapped adjacent to each other, a reduced set of 275 KASP markers representing the distribution across the map were used for this genotyping. From the three $F_{2:3}$ genotypes obtained, the genotype of the F_2 parent was inferred as follows: if all three genotypes for a marker were homozygous for the same allele, the F_2 parent plant was called homozygous for that allele. Instead, if they were all heterozygous or segregated, the F_2 parent plant was called heterozygous. Alleles for each SNP were scored as heterozygous ('h'), homozygous to parent P20 ('a') or homozygous to parent P22 ('b'). Marker

segregation was analysed for conformity to Mendelian ratios expected in an F_2 population using a chi-squared test. Markers were initially grouped based on independence logarithm of odds (LOD) scores >5.0 using the G^2 statistic as calculated by JoinMap®4 for recombination frequency (Van Ooijen, 2006). Markers within groups were then ordered using the regression mapping algorithms as described by Stam (1993), with successive rounds of marker placement utilised to add loci to the map, with a maximum of three rounds.

5.2.9 QTL Analysis

The molecular marker linkage map for the KASPar™ SNPs (Section 5.4.2) was used as a framework for the QTL analysis. Interval mapping was conducted using R/qtl (Broman *et al.*, 2003) to increase resolution and reduce background marker effects. QTL were detected with QTL significance at $p<0.05$ using a genome wide threshold for LOD. Additional MQM mapping was carried out using MapQTL®6.0 (Van Ooijen, 2006; Jansen 1993, 1994; and Jansen and Stam 1994) to identify cofactors to improve the significance of the 95% LOD threshold. A permutation test with 1,000 iterations was used to determine the 95% LOD significance thresholds for detecting QTL at the individual linkage group and genome wide levels.

5.3 Results

5.3.1 Parent line root assay to screen for resistance to *I. pastinacae*

Following inoculation with *I. pastinacae* isolate IP10, all parsnip parent line roots developed lesions (Fig 5.1), with $\log_e$-transformed mean lesion areas ranging from 0.2 cm^2 to 1.2 cm^2 (Fig 5.2). The ANOVA showed change in parsnip line to be a significant effect on lesion area ($p<0.05$), indicating that there is a significant genetic component to the variation. Based on the LSD (5%) value of 0.58, the lines are separated into three groups (Table 5.3), where each distinct group is significantly different ($p<0.05$) to other groups. The highest lesion area was seen in line P25, which is significantly different ($p<0.05$) from seven other parent lines. The lowest lesion area was displayed in line P14, which is grouped with six other parent lines displaying low lesion areas; these lines are significantly different ($p<0.05$) to P25 only. The intermediate lines, P11 and P22, overlap with both LSD calculated groups 'a' and 'b', so are not significantly different ($p>0.05$) to any other line.

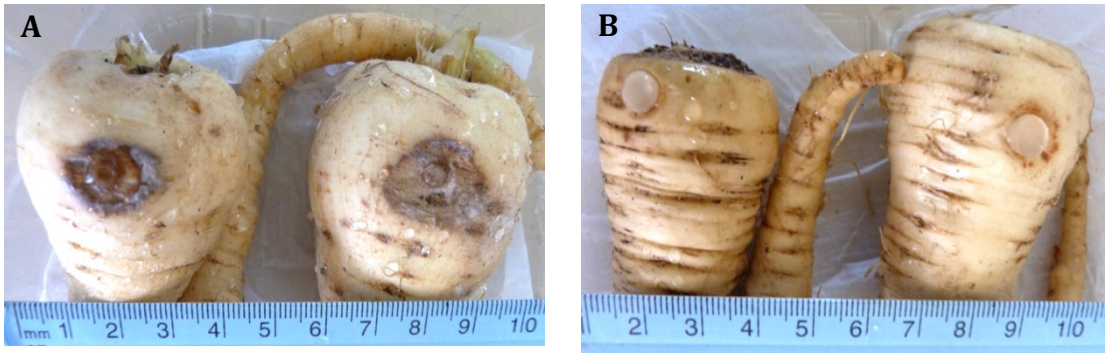


Figure 5.1 – Developing symptom of *I. pastinacae* isolate IP10 inoculated parent line parsnip roots 50 days after inoculation with 5 mm agar plug. A) Parent line P25, most susceptible, and B) parent line P14, most resistant.

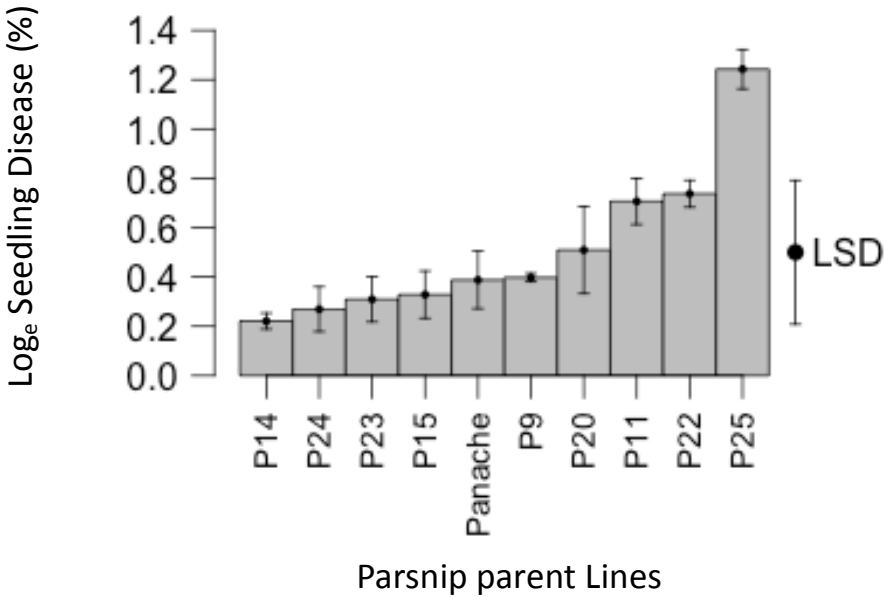


Figure 5.2 – Log$_e$ root lesion area (cm^2) of 10 parsnip parent lines inoculated with *I. pastinacae* isolate IP10. Error bars show SEM from three independent replicates alongside the LSD (5% level).

Table 5.3 – Log$_e$-transformed means, 5% LSD value and corresponding significance group based on *I. pastinacae* isolate IP10 inoculation of parsnip parent line roots. The degrees of freedom (d.f.) were 9.

Number	Line	Log$_e$-transformed mean	5% LSD
1	P25	1.2697416	a
2	P22	0.7518508	ab
3	P11	0.7469382	ab
4	P20	0.5776422	b
5	Panache	0.436948	b
6	P9	0.4094555	b
7	P15	0.3693819	b
8	P23	0.3402982	b
9	P24	0.3016111	b
10	P14	0.2241443	b
d.f.	9		
5% LSD	0.5837355		

5.3.2 Parent line root assay to screen for resistance to *M. acerina*

Following inoculation with *M. acerina* isolate MA2, all parsnip parent lines developed lesions (Fig 5.3), with $\log_e$-transformed means ranging from -0.5 cm^2 to 1.25 cm^2 (Fig 5.4). The ANOVA indicated parent line had a significant effect on lesion area ($p<0.001$). The LSD (5%) value of 0.689 separates the parsnip parent lines into seven groups (Table 5.4), where each distinct group is significantly different ($p<0.05$) to other groups. The highest lesion area was observed in line P22, which is significantly different ($p<0.05$) from six other lines. The lowest lesion area is observed in line Panache, which is significantly different ($p<0.05$) from six other lines. The remaining intermediate isolates are significantly different ($p<0.05$) from at least two other lines.

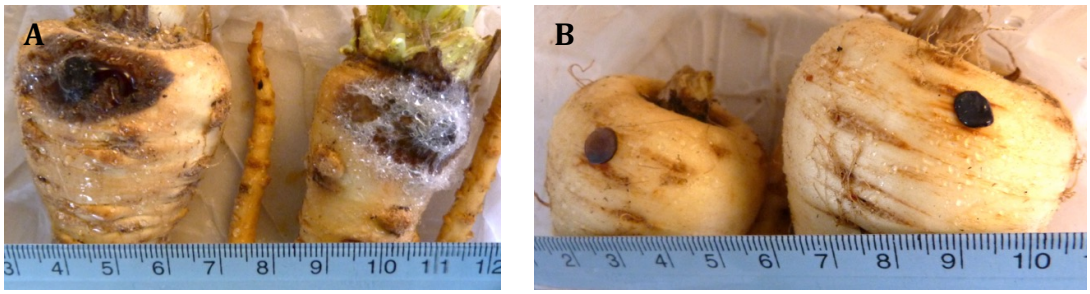


Figure 5.3 – Developing symptom of *M. acerina* isolate MA2 inoculated parent line parsnip roots 30 days after inoculation with a 5 mm agar plug. A) Parent line P22, most susceptible, and B) parent line Panache, most resistant.

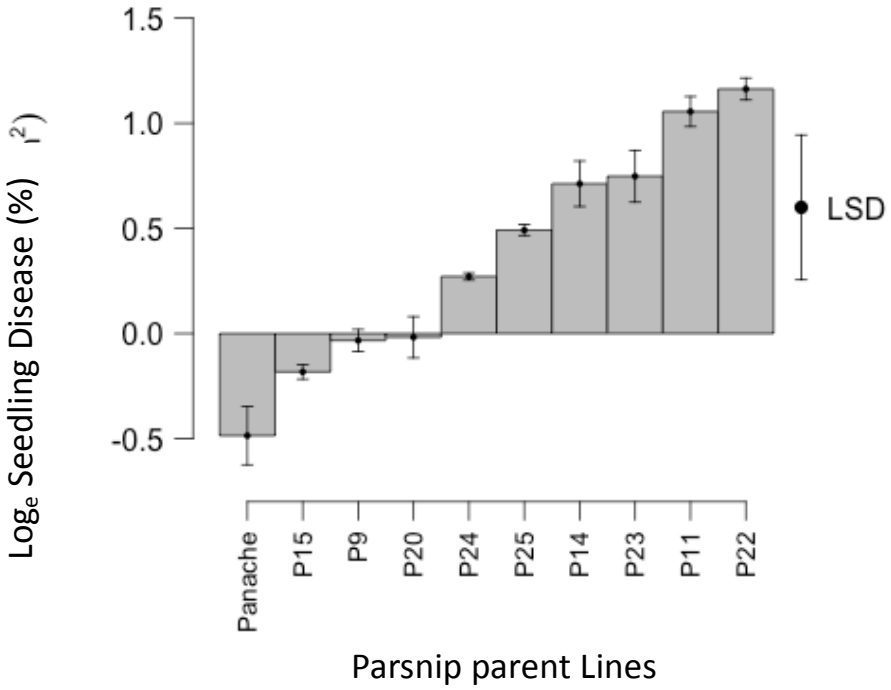


Figure 5.4 – Log_e root lesion area (cm^2) of 10 parsnip parent lines inoculated with *M. acerina* isolate MA2. Error bars show SEM from three independent replicates alongside the LSD (5% level).

Table 5.4 – Log_e-transformed means, 5% LSD value and corresponding significance group based on *M. acerina* isolate MA2 inoculation of parsnip Parent Line roots. The degrees of freedom (d.f.) were 9.

Number	Line	Log_e-transformed mean	5% LSD
1	P22	1.192005077	a
2	P11	1.075830963	ab
3	P14	0.793291126	abc
4	P23	0.788043412	abc
5	P25	0.496198499	bcd
6	P24	0.273245333	cd
7	P20	0.040119743	de
8	P9	-0.003466196	de
9	P15	-0.168720075	de
10	Panache	-0.438990708	e
d.f.	9		
5% LSD	0.6890569		

5.3.3 Parent line root assay to screen for resistance to *C. destructans*

Following inoculation with *C. destructans* isolate CD28, all parsnip parent lines developed lesions (Fig 5.5), with $\log_e$-transformed means ranging from -0.2 cm^2 to 1.0 cm^2 (Fig 5.6). The ANOVA showed Parent line to have a significant effect on lesion area ($p<0.05$). The LSD (5%) value of 0.464 separates the parsnip parent lines into four groups (Table 5.5). The highest lesion area was observed in line P23, which is significantly different ($p<0.05$) from all other parsnip parent lines. The lowest lesion area is observed in line P15, which is also significantly different ($p<0.05$) from all other parsnip parent lines. The eight intermediate isolates are significantly different ($p<0.05$) from one or two other parsnip parent lines, due to overlapping LSD groups.

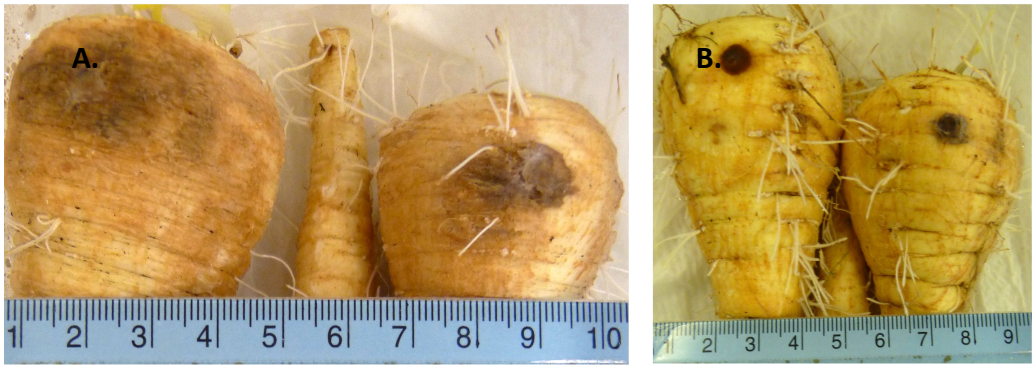


Figure 5.5 – Developing symptom of *C. destructans* isolate CD28 inoculated parent line parsnip roots 30 days after inoculation with a 5 mm agar plug. A) Parent line P23, most susceptible, and B) parent line P15, most resistant.

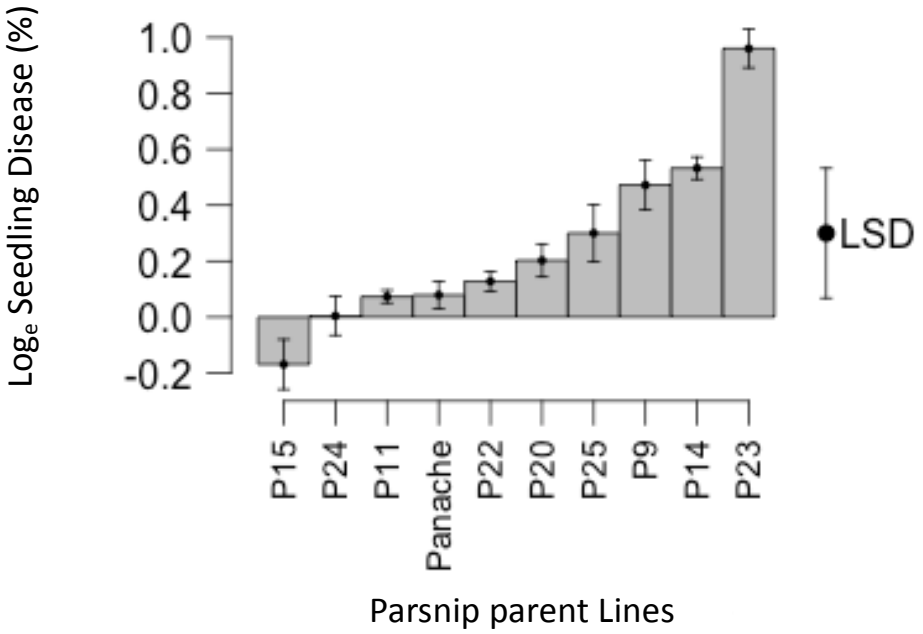


Figure 5.6 – Log$_e$ root lesion area (cm^2) of 10 parsnip parent lines inoculated with *C. destructans* isolate CD28. Error bars show SEM from three independent replicates alongside the LSD (5% level).

Table 5.5 – Log$_e$ transformed means, 5% LSD value and corresponding significance group based on *C. destructans* isolate CD28 inoculation of parsnip parent line roots. The degrees of freedom (d.f.) were 9.

Number	Line	Log$_e$-transformed mean	5% LSD
1	P23	1.0417074	a
2	P9	0.5662831	b
3	P14	0.5511062	b
4	P25	0.4085262	b
5	P20	0.2690951	bc
6	P22	0.1584034	bc
7	P24	0.1542320	bc
8	Panache	0.1377559	bc
9	P11	0.1125462	bc
10	P15	0.1014961	c
d.f.	9		
5% LSD	0.4642377		

5.3.4 Parent line seedling assay to screen for resistance to *I. pastinacae*

Inoculation of parsnip seed with *I. pastinacae* isolate IP10 caused seedling death and brown root symptoms to develop across all parent lines (Fig 5.7). As *I. pastinacae* is a seed-borne pathogen, background rates are present on the non-inoculated seed, leading to seedling death and brown root symptoms in the control groups. The mean percentage of infected seedlings ranged from 30% to 80% in inoculated parent lines, and 5% to 20% in the control, non-inoculated, lines (Fig 5.8). Parent line P15 displayed the highest mean seedling disease incidence in inoculated seed, but the lowest mean seedling disease incidence in control seed, whilst line P20 exhibits the lowest seedling death in inoculated seed but one of the highest mean seedling disease incidences of the control seed.

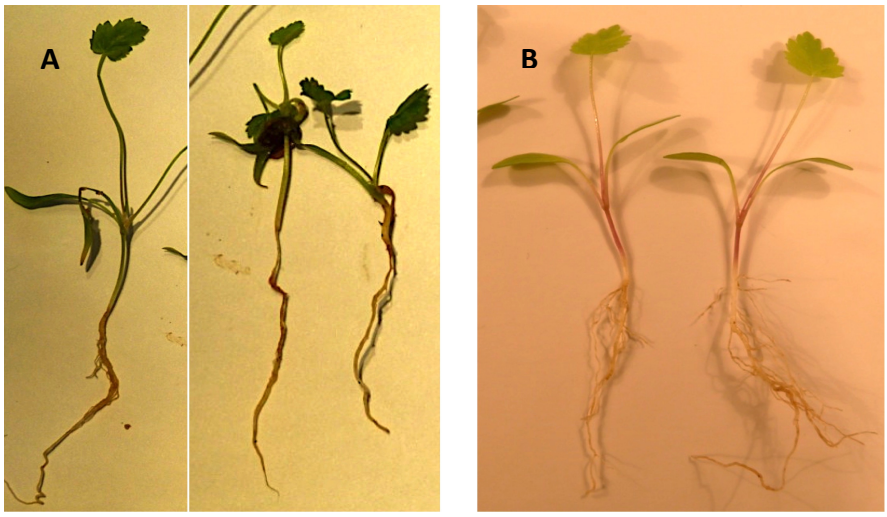


Figure 5.7 – Brown root symptoms on parsnip parent line seedlings from seed inoculated with *I. pastinacae* isolate IP10. A) Parent line Panache seedling with brown root symptoms and B) parent line Panache with no brown root symptoms.

A significant difference between inoculated and control seed ($p<0.05$) was found using a GLM. The parent lines show a significant effect ($p<0.05$) on seedling disease so a posthoc 'Tukey' analysis (Table 5.6) was performed to highlight significant differences between individual control and inoculated lines. The analysis of inoculated lines found P20 to be significantly different ($p<0.05$) to all other parent lines other than P22. Line P22 was significantly different ($p<0.05$) from P9 and P15, the two lines displaying the highest seedling disease. In the control lines, P15 was significantly different ($p<0.05$) to all other lines, P9 was significantly different to five other lines and significant differences were identified between P24 and P22 and Panache with P14 and P15.

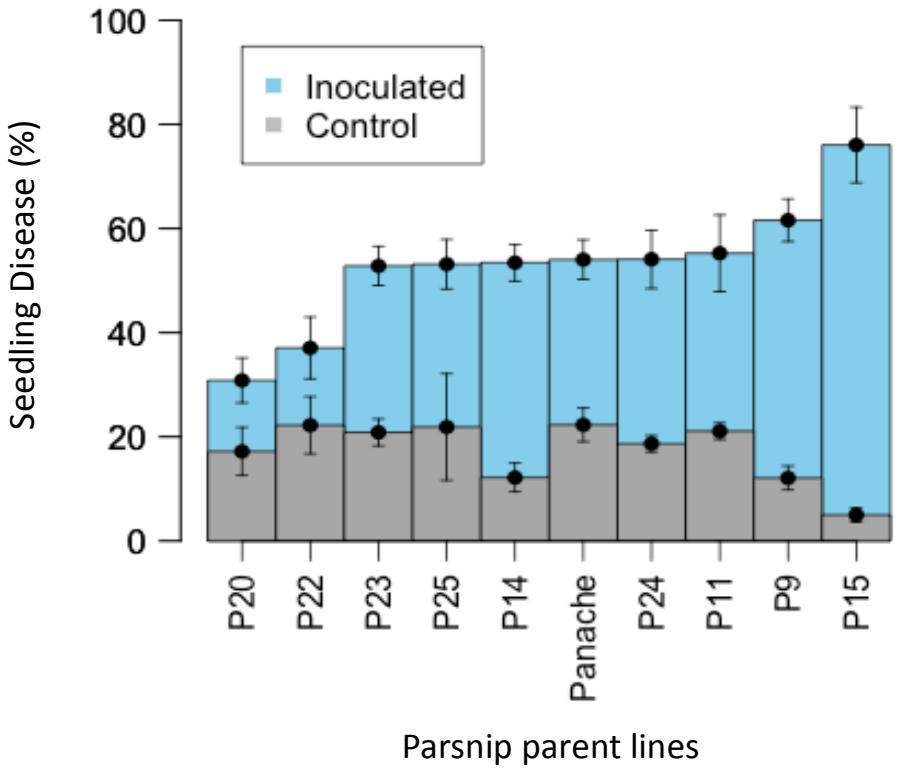


Figure 5.8 – Ranked final seedling disease (%), including death of *I. pastinacae* isolate IP10 inoculated and control parsnip parent line seedlings. Blue bars represent inoculated seed, grey bars indicate control seed. Note that bars are not stacked but overlaid, e.g. the percentage seedling disease for P20 inoculated seed is 30%. Error bars show SEM from four complete replicates.

Table 5.6 – Summary of 'Tukey' multicomparison posthoc test of significance for all parsnip parent lines. A) Seed inoculated with *I. pastinacae* isolate IP10. B) Control seed. Yellow indicates significant difference of p<0.05, orange of p<0.01 and red of p<0.001.

A	P20	P22	P23	P25	P14	Panache	P24	P11	P9	P15
P20										
P22										
P23										
P25										
P14										
Panache										
P24										
P11										
P9										
P15										

B	P20	P22	P23	P25	P14	Panache	P24	P11	P9	P15
P20										
P22										
P23										
P25										
P14										
Panache										
P24										
P11										
P9										
P15										

5.3.5 Parent line seedling assay to screen for resistance to *M. acerina*

M. acerina isolate MA2 inoculum caused seedling death and brown root symptoms to develop across all parsnip parent lines. *M. acerina* is a soil-borne pathogen, with limited evidence of being seed-borne on hosts unrelated to parsnip. The seedling disease and death observed in the control lines is predominantly due to poor germination and vigour in parsnips, leading to increased seedling damping-off. The mean seedling disease incidence in inoculated lines ranged from 75% to 95%, and from 2% to 20% in the control lines (Fig 5.9). Parent line P24 displayed the highest seedling disease (%), and P15 the lowest seedling disease. The highest seedling disease (%) in the control lines was observed in P25 with the lowest in P15. Parent line P15 displayed the lowest seedling disease in both the inoculated and controls, with P24 displaying the highest seedling disease in the inoculated and one of the highest in the control.

A GLM showed there to be a significant difference ($p<0.05$) between the inoculated and control groups, with parent line to have a significant effect ($p<0.05$) on seedling disease. A posthoc 'Tukey' analysis (Table 5.7) identified significant differences between individual lines from both the control and inoculated group. The analysis of the inoculated lines identified a significant difference ($p<0.001$) between P15 and P24, the lines with highest and lowest severity of seedling disease. More differences were identified within the control group, with P15 being significantly different ($p<0.05$) to all other lines apart from P22. Lines P23 and P25 displayed significant differences ($p<0.05$) from six other lines, whilst P22 and P24 displayed significant differences from three and four other lines, respectively.

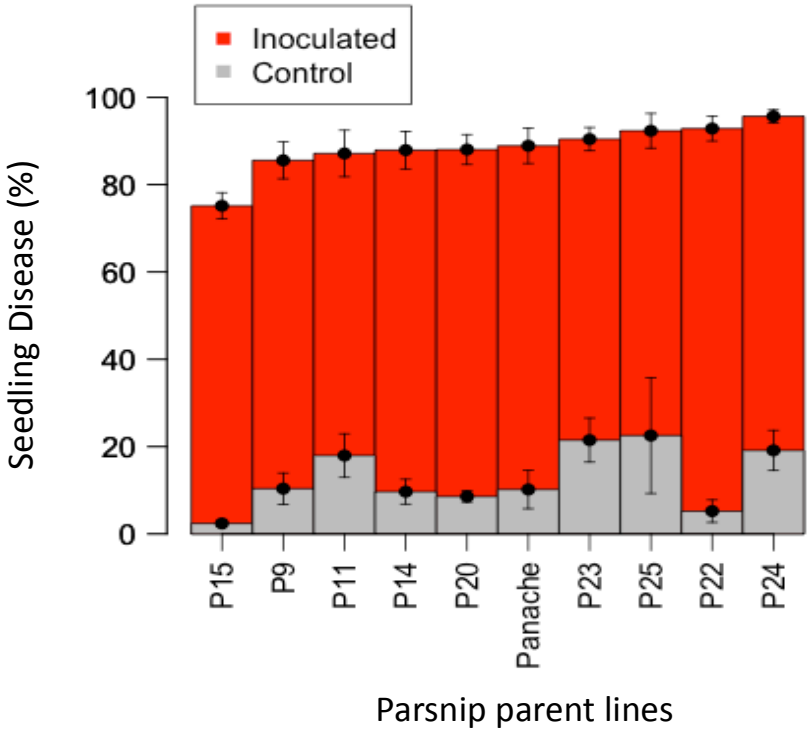


Figure 5.9 – Ranked final seedling disease (%), including death of *M. acerina* isolate MA2 inoculated and control parsnip Parent line seedlings. Red bars represent inoculated seed, grey bars indicate control seed. Note that bars are not stacked but overlaid, e.g. the percentage seedling disease for P15 inoculated seed is 76%. Error bars show SEM from four complete replicates.

Table 5.7 – Summary of 'Tukey' multicomparison posthoc test of significance for all parsnip Parent lines. A) Seed inoculated with *M. acerina* isolate MA2. B) Control seed. Yellow indicates significant difference of p<0.05, orange of p<0.01 and red of p<0.001.

A	P15	P9	P11	P14	P20	Panache	P23	P25	P22	P24
P15										
P9										
P11										
P14										
P20										
Panache										
P23										
P25										
P22										
P24										

B	P15	P9	P11	P14	P20	Panache	P23	P25	P22	P24
P15										
P9										
P11										
P14										
P20										
Panache										
P23										
P25										
P22										
P24										

5.3.6 Parent line seedling assay to screen for resistance to *C. destructans*

C. destructans isolate CD28 inoculum caused seedling death and brown root symptoms to develop across all parsnip parent lines, including both inoculated and control groups. The most likely explanation for any apparent seedling disease observed in the control groups is the known low germination and poor seedling vigour of parsnip. The mean seedling disease incidence within the inoculated lines range from 65% to 95%, while the mean of the control lines ranged from 0% to 5% (Fig 5.10). Within the inoculated lines, P11 displayed the highest seedling disease (%) and P20 the lowest. The control line P9 exhibited the highest seedling disease (%), with lines Panache, P24, P15, P25 and P23 equally displaying the lowest seedling disease (0%).

A GLM analysis showed a significant difference ($p<0.05$) between the inoculated and control groups, and showed line to have a significant effect ($p<0.05$) on seedling disease. The posthoc 'Tukey' analysis identified no significant differences between inoculated lines. Within the control lines, there were only three significant differences ($p<0.05$), between P9 and, respectively, Panache, P14 and P22 (Table 5.8).

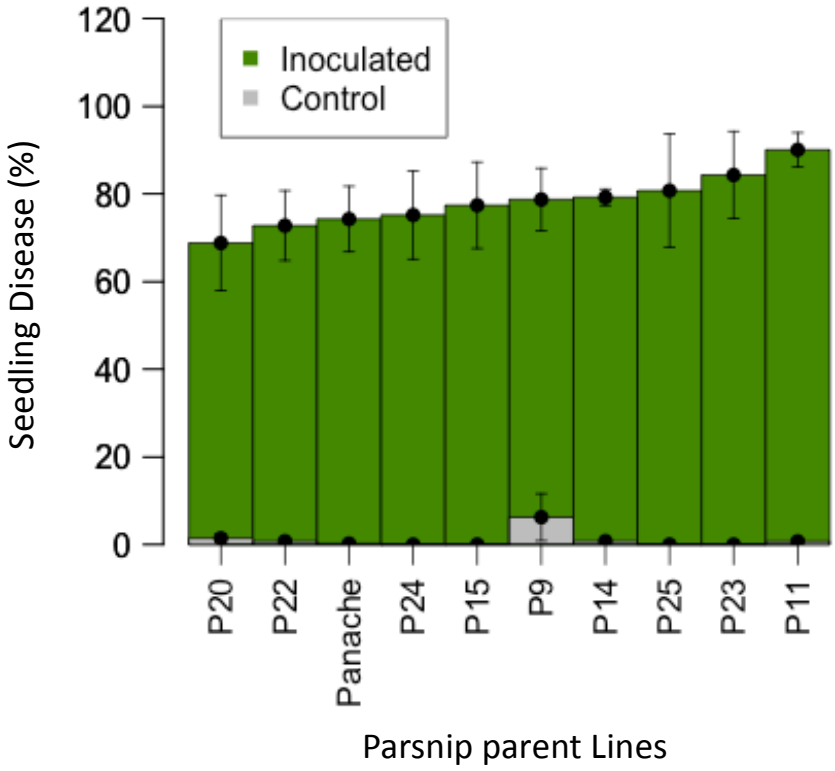


Figure 5.10 – Ranked final seedling disease percentage, including death of *C. destructans* isolate CD28 inoculated and control parsnip parent line seedlings. Green bars represent inoculated seed, grey bars indicate control seed. Note that bars are not stacked but overlaid, e.g. the percentage seedling disease for P20 inoculated seed is 68%. Error bars show SEM from four complete replicates.

Table 5.8 – Summary of 'Tukey' multicomparison posthoc test of significance for all parsnip parent lines in *C. destructans* resistance assay. Yellow indicates significant differences of $p<0.05$. Only control lines are compared, as no significant differences were detected between inoculated lines.

A	P20	P22	Panache	P24	P15	P9	P14	P25	P23	P11
P20										
P22										
Panache										
P24										
P15										
P9										
P14										
P25										
P23										
P11										

5.3.7 Breeding line root assay to screen for resistance to *I. pastinacae*

Following inoculation with *I. pastinacae* isolate IP10, all parsnip breeding line roots developed lesions, with $\log_{10}$ transformed mean lesion area ranging from -0.21 cm^2 to 0.72 cm^2 (Fig 5.11). The ANOVA showed line to be a significant effect on lesion area ($p<0.05$). Based on the LSD (5%) value of 0.2536, the lines are separated into 18 groups (Table 5.9), where each distinct group is significantly different ($p<0.05$) to other groups. The highest lesion area was seen in line B3, which is significantly different ($p<0.05$) from 16 other breeding lines. The two lowest lesion areas are observed in breeding lines B22 and B21, each of which is significantly different ($p<0.05$) from all other lines.

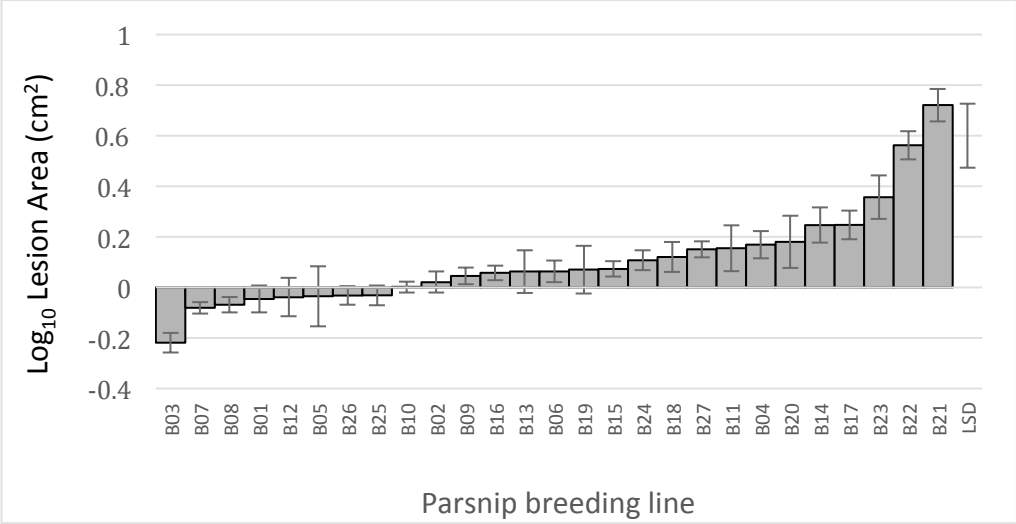


Figure 5.11 – Log_{10}-transformed root lesion area (cm^2) of 27 parsnip breeding lines inoculated with *I. pastinacae* isolate IP10. Error bars show SEM from three replicates, alongside the LSD (5% level).

Table 5.9 – Log_{10}-transformed means, 5% LSD value and corresponding significance group based on *I. pastinacae* isolate IP10 inoculation of parsnip breeding line roots. The degrees of freedom (d.f.) were 26.

Number	Breeding line	Log_{10}-transformed mean	5% LSD
1	B21	0.7215	a
2	B22	0.5625	ab
3	B23	0.3569	abc
4	B17	0.2474	abcd
5	B14	0.2465	abcd
6	B20	0.1803	abcd
7	B04	0.1693	abcde
8	B11	0.1552	abcdef
9	B27	0.1509	bcdef
10	B18	0.1204	abcdefg
11	B24	0.1074	cdefgh
12	B15	0.0731	cdefgh
13	B19	0.0707	bcdefghi
14	B06	0.0633	cdefghij
15	B13	0.0632	bcdefghijk
16	B16	0.0579	cdefghijkl

Number	Breeding line	Log_{10}-transformed mean	5% LSD
17	B09	0.0455	cdefghijklm
18	B02	0.0206	dghijklm
19	B10	0.001	giklm
20	B25	-0.0312	defghijklmn
21	B26	-0.0321	ghijklmno
22	B05	-0.0348	dfghijklmnop
23	B12	-0.039	ikmnop
24	B01	-0.0458	ikmnop
25	B08	-0.0685	np
26	B07	-0.0806	q
27	B03	-0.2186	r
d.f.	26		
5% LSD	0.2536		

5.3.8 Breeding line root assay to screen for resistance to *M. acerina*

Following inoculation with *M. acerina* isolate MA2, all parsnip breeding line roots developed lesions, with mean lesion area ranging from 2.16 cm^2 to 6.44 cm^2 (Fig 5.12). The ANOVA showed line to be a significant effect on lesion area ($p<0.05$). Based on the LSD (5%) value of 1.926, the lines are separated into nine groups (Table 5.10), where each distinct group is significantly different ($p<0.05$) to other groups. The highest lesion area was seen in line B21, and the lowest lesion area in line B26. The LSD (5%) shows all groups overlapping, indicating only lines in the highest and lowest end of the spectrum of lesion areas were significantly different from each other.

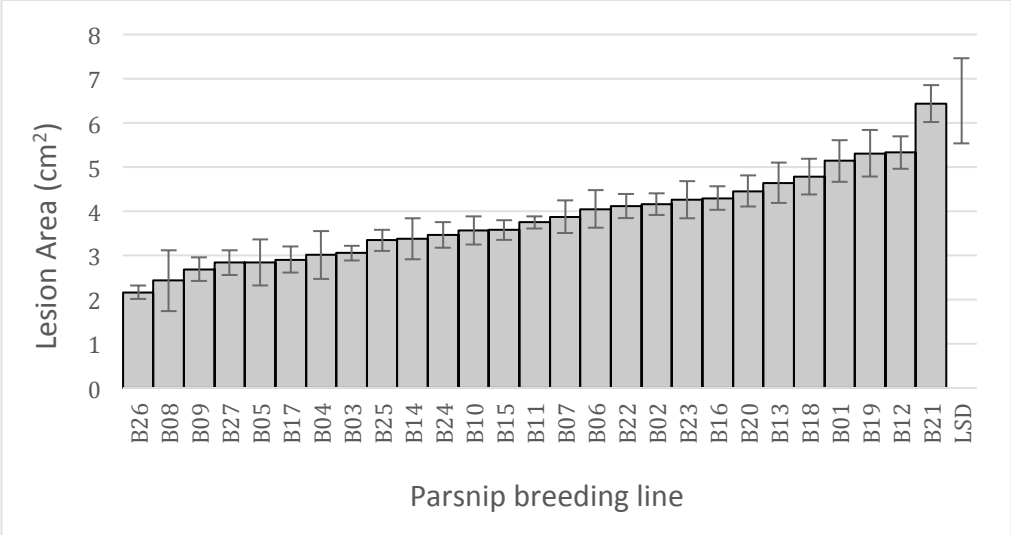


Figure 5.12 – Lesion area (cm^2) of 27 parsnip breeding line roots inoculated with *M. acerina* isolate MA2. Error bars show SEM from three replicates, alongside the LSD (5% level).

Table 5.10 – Means, 5% LSD value and corresponding significance group based on *M. acerina* isolate MA2 inoculation of parsnip breeding line roots. The degrees of freedom (d.f.) were 26.

Number	Line	Mean	5% LSD
1	B21	6.442	a
2	B12	5.329	ab
3	B19	5.313	ab
4	B01	5.141	abc
5	B18	4.789	abcd
6	B13	4.644	abcde
7	B20	4.458	abcdef
8	B16	4.297	abcdef
9	B23	4.262	abcdef
10	B02	4.163	abcdef
11	B22	4.12	abcdef
12	B06	4.051	abcdef
13	B07	3.877	abcdef
14	B11	3.75	abcdef
15	B15	3.577	abcdef

Number	Line	Mean	5% LSD
16	B10	3.567	abcdef
17	B24	3.459	abdef
18	B14	3.373	abcdefg
19	B25	3.344	abcdefgh
20	B03	3.054	abdefgh
21	B04	3.013	abcdefghi
22	B17	2.907	abcdefghi
23	B05	2.843	bdefghi
24	B27	2.84	bdefghi
25	B09	2.688	bdefghi
26	B08	2.431	bdfghi
27	B26	2.163	gi
d.f.	26		
5% LSD	1.926		

5.3.9 Genotyping population root assay to screen for resistance to *I. pastinacae*

Following inoculation with *I. pastinacae* isolate IP10, all 2013/14 genotyping population roots developed lesions, with log_{10} adjusted mean lesion area ranging from -0.8 cm^2 to 0.3 cm^2 (Fig 5.13A). The log_{10}-transformed mean values were adjusted through REML analysis to counter for significant random effects within the REML model. The REML analysis identified replicate to be a significant effect ($p<0.001$, Wald statistic = 83.84) (Table 5.11), so adjusted means were generated. The inoculation of the genotyping population with *I. pastinacae* isolate IP10 in the year 2014/15 also led to the development of lesions on all roots. The log_{10}-transformed means were again adjusted through REML analysis, as replicate was found to be a significant effect within the model ($p<0.001$, Wald statistic = 18.62) (Table 5.11). The log_{10}-transformed REML adjusted means for 2014/15 ranged from -0.5 cm^2 to 0.5 cm^2 (Fig 5.13B).

From the REML analysis of both the 2013/14 and 2014/15 datasets showed line, the fixed effect, to be significant ($p<0.001$, Wald statistic = 194.79 and $p<0.001$, Wald statistic = 365.48) on lesion area (Table 5.11). This verifies that there is significant genetic variation that can be used in QTL analysis to search for resistance loci. A significant difference ($p<0.5$) was also identified between the years 2013/14 and 2014/15, although some lines did remain in a similar position across both datasets, e.g. in the case of G58, which displayed high lesion areas in both datasets.

Following REML analysis, the 5% LSD was calculated to directly compare lines; in both the 2013/14 and 2014/15 populations, a significant difference to the 5% LSD was identified between the lines with the highest and lowest lesion areas only. Full 5% LSD outputs can be found in the appendix (Tables A.9 and A.10).

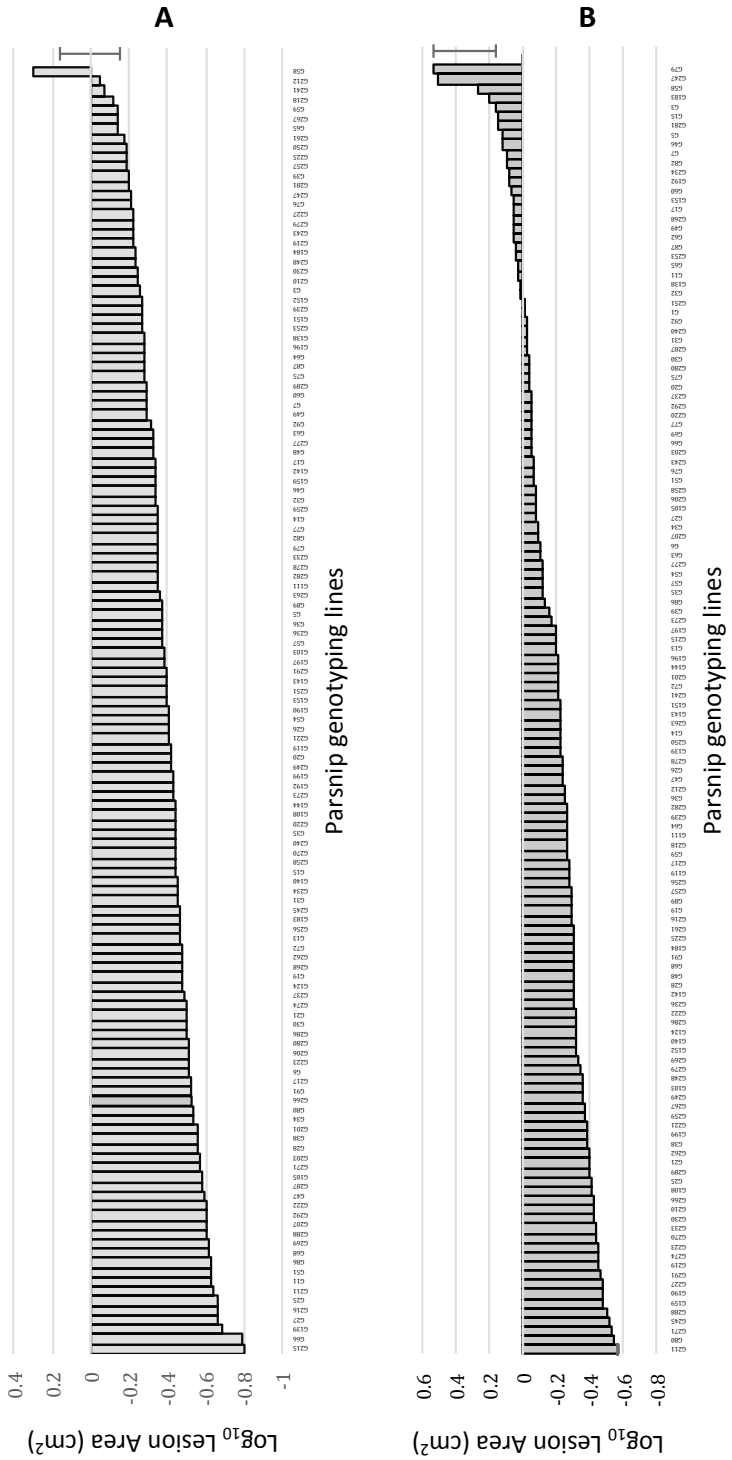


Figure 5.13 – Log_{10} lesion area of (A) 135 parsnip genotyping lines from the year 2013/14 and (B) 138 parsnip genotyping lines from the year 2014/15, inoculated with *M. acerina* isolate MA2. Bars represent log_{10}-transformed adjusted mean values from REML analysis of 3 independent replicates. Error bar represents the LSD (5%) level.

Table 5.11 – REML analysis outputs from log_{10}-transformed adjusted means from *I. pastinacae* and *M. acerina* inoculated genotype population roots over three years. Wald statistic, p-value, variance and standard deviation (SD) given for random effects (replicate (rep) and box) of the REML and the Wald statistic, degrees of freedom (d.f.) and Chi-square p-value for the fixed effect (Genotype line) of the REML analysis.

Phenotype		Random effect					Fixed effect			
			REML Wald statistic	REML p-value	Variance	SD		REML Wald statistic	d.f.	Chi-Sq p-value
***I. pastinacae* 2013/14**		**Rep**	83.84	<0.001	0.007509	0.08665	**Line**	194.79	134	<0.001
		Box	0.22	0.641	<0.001	<0.001				
***I. pastinacae* 2014/15**		**Rep**	18.62	<0.001	0.0001305	0.01143	**Line**	365.48	137	<0.001
		Box	0.01	0.911	<0.001	<0.001				
***M. acerina* 2013/14**		**Rep**	57.24	<0.001	1.342×10^{-4}	0.011583	**Line**	297.25	135	<0.001
		Box	0.05	0.713	5.908×10^{-5}	0.007686				
***M. acerina* 2014/15**		**Rep**	47.95	<0.001	0.02384	0.1544	**Line**	316.7	137	<0.001
		Box	0.02	0.421	<0.001	<0.001				
***M. acerina* 2014/15**		**Box**	0.01	<0.001	<0.001	<0.001	**Line**	401.13	126	<0.001

5.3.10 Genotyping population root assay to screen for *resistance to M. acerina*

Following inoculation with *M. acerina* isolate MA2, all 2013/14 genotyping population roots developed lesions. Log_{10}-transformed data was adjusted through REML analysis to counter for any significant random effects within the REML model. The REML model indicated replicate was a significant random effect ($p<0.001$, Wald statistic = 57.24) (Table 5.11). The log_{10} adjusted mean lesion areas range from -0.6 cm^2 to 0.5 cm^2 (Fig 5.14A), with line being a significant factor ($p<0.001$, Wald statistic = 365.48) (Table 5.11), indicating significant genetic variation.

The inoculation of root from the genotyping population with *M. acerina* isolate MA2 for the year 2014/15 also led to the development of lesions on all roots. The log_{10}-transformed means were adjusted through a REML analysis, identifying replicate as a significant random effect ($p<0.001$, Wald statistic = 47.92) (Table 5.11), and range from -0.3 cm^2 to 0.6 cm^2 (Fig 5.14B). The final year, 2015/16 of *M. acerina* isolate MA2 inoculated genotype line roots led to the development of lesions across all roots. The mean lesion areas were log_{10}-transformed and adjusted through REML analysis to counter for significant random effects. The adjusted mean lesion areas range from -0.5 cm^2 to 0.5 cm^2 (Fig 5.14C). For 2013/14, 2014/15 and 2015/16 the REML analysis identified line, the fixed effect, to be a significant factor ($p<0.001$, Wald statistic = 297.25, $p<0.001$, Wald statistic = 316.7 and $p<0.001$, Wald statistic = 401.13 respectively) (Table 5.11). A significant difference was identified between each year of the experiment, 2013/14, 2014/15 and 2015/16; however, there are similarities in some lines between years, such as Line G247 displaying high lesion area in 2014/15 and 2015/16, and G28 displaying small lesion area in both 2013/14 and 2015/16. A post-hoc 5% LSD was calculated, as a multicomparison for each line. This showed a significant different to the 5% LSD to be observed in the lines with the highest and lowest lesion areas across all three years, 2013/14, 2014/15 and 2015/16. Full analysis can be found in the appendix (Tables A.11, A.12 and A.13)..

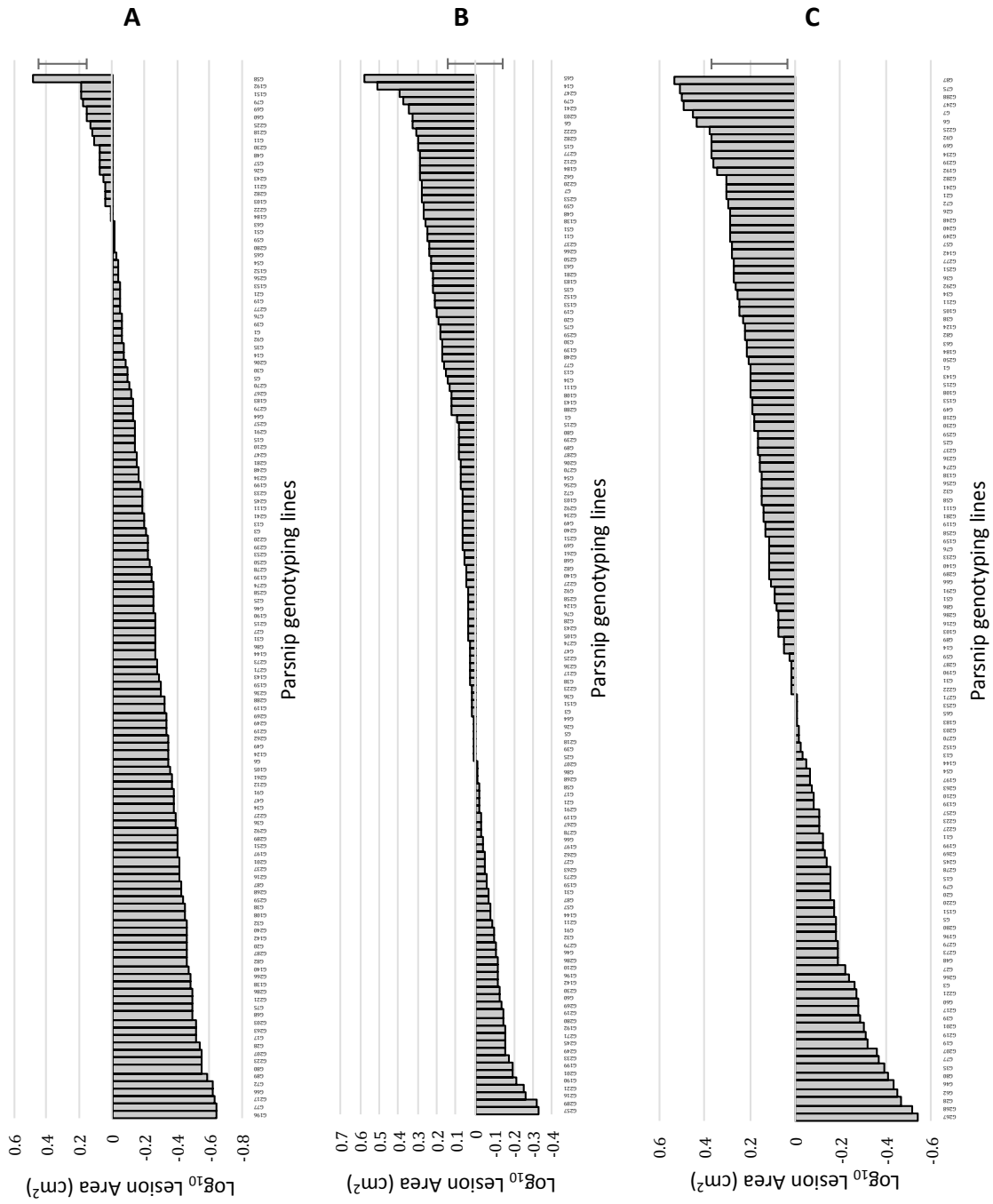
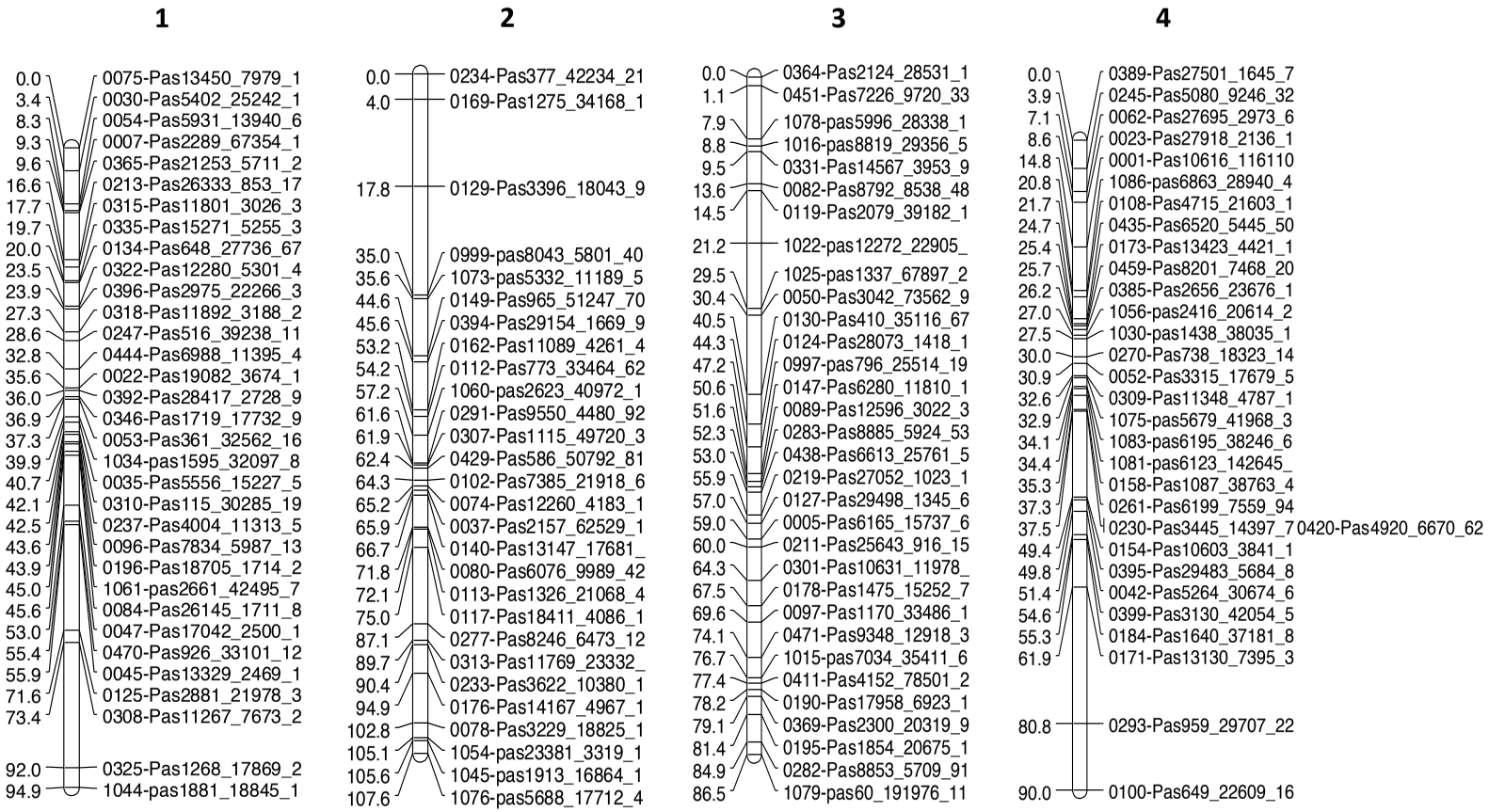


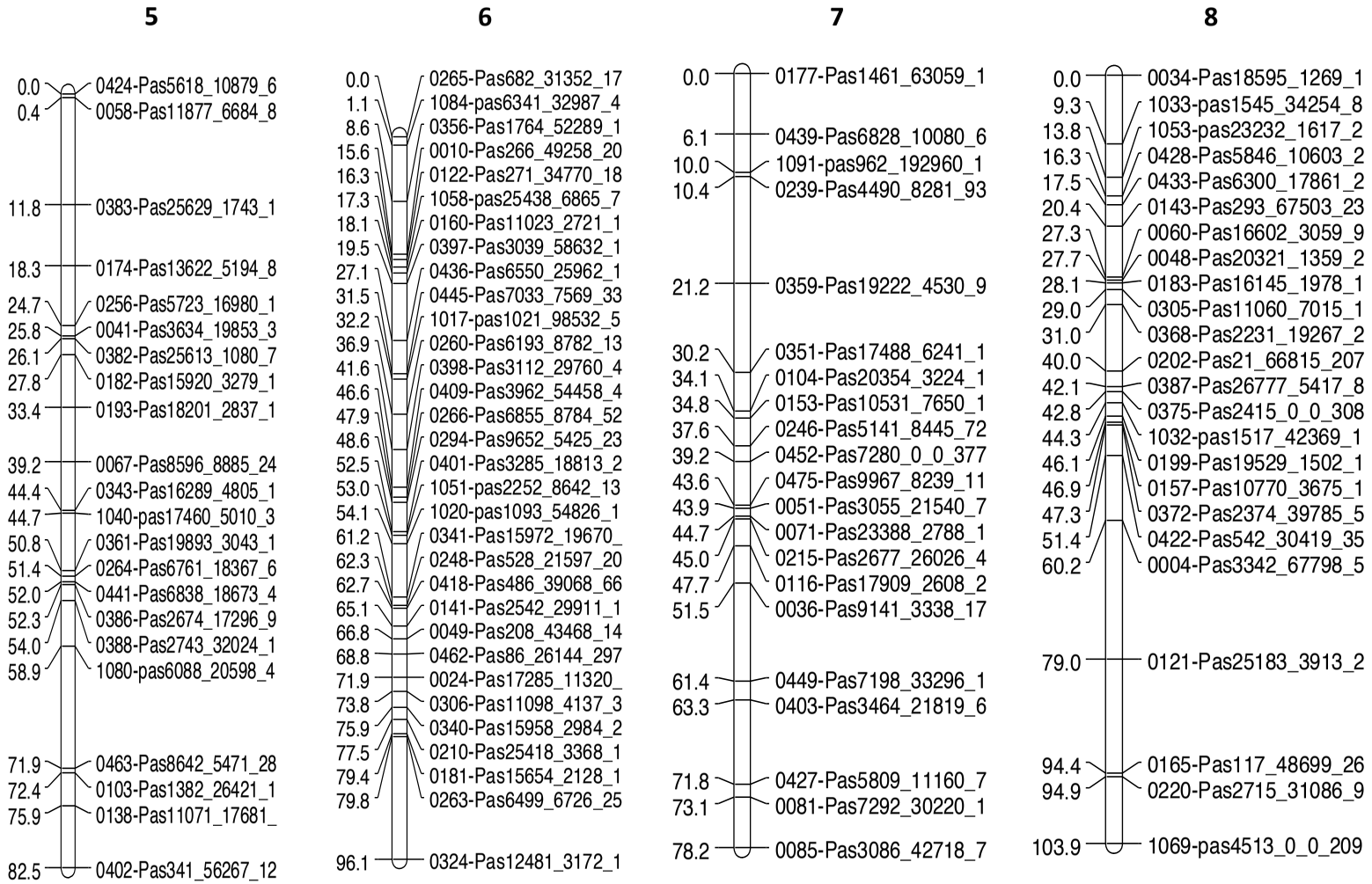
Figure 5.14 – Log_{10} lesion area of (A) 136 parsnip genotyping lines from the year 2013/14, (B) 138 parsnip genotyping lines from the year 2014/15, and (C) 128 parsnip genotyping lines from the year 2015/16, inoculated with *M. acerina* isolate MA2. Bars represent log_{10}-transformed adjusted mean values from REML analysis of 3 independent replicates. Error bar represents the LSD (5%) level.

5.3.11 Linkage map construction

The parents of the mapping population, P20 x P22 were chosen by Elsoms Seeds Ltd as disease resistant and disease susceptible parents to produce a segregating population for QTL analysis. A total of 274 SNP loci were genotyped using the KASPar^{TM} genotyping chemistry and included in the linkage map.

Pairwise linkage analysis grouped all 274 markers into 11 linkage groups, corresponding to each of the *P. sativa* haploid chromosomes (2n = 22). The distribution of markers within the linkage groups varies from 14 to 33 SNPs per linkage group (Table 5.12). Regression mapping of the pairwise linkage groups successfully ordered all SNP markers within their respective linkage groups. The centiMorgan (cM) distance, spanned by the SNP markers in the linkage groups ranged from 0 to 107.6 cM. The total map consists of 274 SNP loci and spanned a total of 1005 cM. The largest interval between two markers was 26 cM on linkage group 10 (Fig 5.18).


1
0.0 0075-Pas13450_7979_1
3.4 0030-Pas5402_25242_1
8.3 0054-Pas5931_13940_6
9.3 0007-Pas2289_67354_1
9.6 0365-Pas21253_5711_2
16.6 0213-Pas26333_853_17
17.7 0315-Pas11801_3026_3
19.7 0335-Pas15271_5255_3
20.0 0134-Pas648_27736_67
23.5 0322-Pas12280_5301_4
23.9 0396-Pas2975_22266_3
27.3 0318-Pas11892_3188_2
28.6 0247-Pas516_39238_11
32.8 0444-Pas6988_11395_4
35.6 0022-Pas19082_3674_1
36.0 0392-Pas28417_2728_9
36.9 0346-Pas1719_17732_9
37.3 0053-Pas361_32562_16
39.9 1034-pas1595_32097_8
40.7 0035-Pas5556_15227_5
42.1 0310-Pas115_30285_19
42.5 0237-Pas4004_11313_5
43.6 0096-Pas7834_5987_13
43.9 0196-Pas18705_1714_2
45.0 1061-pas2661_42495_7
45.6 0084-Pas26145_1711_8
53.0 0047-Pas17042_2500_1
55.4 0470-Pas926_33101_12
55.9 0045-Pas13329_2469_1
71.6 0125-Pas2881_21978_3
73.4 0308-Pas11267_7673_2
92.0 0325-Pas1268_17869_2
94.9 1044-pas1881_18845_1
2
0.0 0234-Pas377_42234_21
4.0 0169-Pas1275_34168_1
17.8 0129-Pas3396_18043_9
35.0 0999-pas8043_5801_40
35.6 1073-pas5332_11189_5
44.6 0149-Pas965_51247_70
45.6 0394-Pas29154_1669_9
53.2 0162-Pas11089_4261_4
54.2 0112-Pas773_33464_62
57.2 1060-pas2623_40972_1
61.6 0291-Pas9550_4480_92
61.9 0307-Pas1115_49720_3
62.4 0429-Pas586_50792_81
64.3 0102-Pas7385_21918_6
65.2 0074-Pas12260_4183_1
65.9 0037-Pas2157_62529_1
66.7 0140-Pas13147_17681_
71.8 0080-Pas6076_9989_42
72.1 0113-Pas1326_21068_4
75.0 0117-Pas18411_4086_1
87.1 0277-Pas8246_6473_12
89.7 0313-Pas11769_23332_
90.4 0233-Pas3622_10380_1
94.9 0176-Pas14167_4967_1
102.8 0078-Pas3229_18825_1
105.1 1054-pas23381_3319_1
105.6 1045-pas1913_16864_1
107.6 1076-pas5688_17712_4
3
0.0 0364-Pas2124_28531_1
1.1 0451-Pas7226_9720_33
7.9 1078-pas5996_28338_1
8.8 1016-pas8819_29356_5
9.5 0331-Pas14567_3953_9
13.6 0082-Pas8792_8538_48
14.5 0119-Pas2079_39182_1
21.2 1022-pas12272_22905_
29.5 1025-pas1337_67897_2
30.4 0050-Pas3042_73562_9
40.5 0130-Pas410_35116_67
44.3 0124-Pas28073_1418_1
47.2 0997-pas796_25514_19
50.6 0147-Pas6280_11810_1
51.6 0089-Pas12596_3022_3
52.3 0283-Pas8885_5924_53
53.0 0438-Pas6613_25761_5
55.9 0219-Pas27052_1023_1
57.0 0127-Pas29498_1345_6
59.0 0005-Pas6165_15737_6
60.0 0211-Pas25643_916_15
64.3 0301-Pas10631_11978_
67.5 0178-Pas1475_15252_7
69.6 0097-Pas1170_33486_1
74.1 0471-Pas9348_12918_3
76.7 1015-pas7034_35411_6
77.4 0411-Pas4152_78501_2
78.2 0190-Pas17958_6923_1
79.1 0369-Pas2300_20319_9
81.4 0195-Pas1854_20675_1
84.9 0282-Pas8853_5709_91
86.5 1079-pas60_191976_11
4
0.0 0389-Pas27501_1645_7
3.9 0245-Pas5080_9246_32
7.1 0062-Pas27695_2973_6
8.6 0023-Pas27918_2136_1
14.8 0001-Pas10616_116110
20.8 1086-pas6863_28940_4
21.7 0108-Pas4715_21603_1
24.7 0435-Pas6520_5445_50
25.4 0173-Pas13423_4421_1
25.7 0459-Pas8201_7468_20
26.2 0385-Pas2656_23676_1
27.0 1056-pas2416_20614_2
27.5 1030-pas1438_38035_1
30.0 0270-Pas738_18323_14
30.9 0052-Pas3315_17679_5
32.6 0309-Pas11348_4787_1
32.9 1075-pas5679_41968_3
34.1 1083-pas6195_38246_6
34.4 1081-pas6123_142645_
35.3 0158-Pas1087_38763_4
37.3 0261-Pas6199_7559_94
37.5 0230-Pas3445_14397_7 0420-Pas4920_6670_62
49.4 0154-Pas10603_3841_1
49.8 0395-Pas29483_5684_8
51.4 0042-Pas5264_30674_6
54.6 0399-Pas3130_42054_5
55.3 0184-Pas1640_37181_8
61.9 0171-Pas13130_7395_3
80.8 0293-Pas959_29707_22
90.0 0100-Pas649_22609_16


5
0.0 0424-Pas5618_10879_6
0.4 0058-Pas11877_6684_8
11.8 0383-Pas25629_1743_1
18.3 0174-Pas13622_5194_8
24.7 0256-Pas5723_16980_1
25.8 0041-Pas3634_19853_3
26.1 0382-Pas25613_1080_7
27.8 0182-Pas15920_3279_1
33.4 0193-Pas18201_2837_1
39.2 0067-Pas8596_8885_24
44.4 0343-Pas16289_4805_1
44.7 1040-pas17460_5010_3
50.8 0361-Pas19893_3043_1
51.4 0264-Pas6761_18367_6
52.0 0441-Pas6838_18673_4
52.3 0386-Pas2674_17296_9
54.0 0388-Pas2743_32024_1
58.9 1080-pas6088_20598_4
71.9 0463-Pas8642_5471_28
72.4 0103-Pas1382_26421_1
75.9 0138-Pas11071_17681_
82.5 0402-Pas341_56267_12
6
0.0 0265-Pas682_31352_17
1.1 1084-pas6341_32987_4
8.6 0356-Pas1764_52289_1
15.6 0010-Pas266_49258_20
16.3 0122-Pas271_34770_18
17.3 1058-pas25438_6865_7
18.1 0160-Pas11023_2721_1
19.5 0397-Pas3039_58632_1
27.1 0436-Pas6550_25962_1
31.5 0445-Pas7033_7569_33
32.2 1017-pas1021_98532_5
36.9 0260-Pas6193_8782_13
41.6 0398-Pas3112_29760_4
46.6 0409-Pas3962_54458_4
47.9 0266-Pas6855_8784_52
48.6 0294-Pas9652_5425_23
52.5 0401-Pas3285_18813_2
53.0 1051-pas2252_8642_13
54.1 1020-pas1093_54826_1
61.2 0341-Pas15972_19670_
62.3 0248-Pas528_21597_20
62.7 0418-Pas486_39068_66
65.1 0141-Pas2542_29911_1
66.8 0049-Pas208_43468_14
68.8 0462-Pas86_26144_297
71.9 0024-Pas17285_11320_
73.8 0306-Pas11098_4137_3
75.9 0340-Pas15958_2984_2
77.5 0210-Pas25418_3368_1
79.4 0181-Pas15654_2128_1
79.8 0263-Pas6499_6726_25
96.1 0324-Pas12481_3172_1
7
0.0 0177-Pas1461_63059_1
6.1 0439-Pas6828_10080_6
10.0 1091-pas962_192960_1
10.4 0239-Pas4490_8281_93
21.2 0359-Pas19222_4530_9
30.2 0351-Pas17488_6241_1
34.1 0104-Pas20354_3224_1
34.8 0153-Pas10531_7650_1
37.6 0246-Pas5141_8445_72
39.2 0452-Pas7280_0_0_377
43.6 0475-Pas9967_8239_11
43.9 0051-Pas3055_21540_7
44.7 0071-Pas23388_2788_1
45.0 0215-Pas2677_26026_4
47.7 0116-Pas17909_2608_2
51.5 0036-Pas9141_3338_17
61.4 0449-Pas7198_33296_1
63.3 0403-Pas3464_21819_6
71.8 0427-Pas5809_11160_7
73.1 0081-Pas7292_30220_1
78.2 0085-Pas3086_42718_7
8
0.0 0034-Pas18595_1269_1
9.3 1033-pas1545_34254_8
13.8 1053-pas23232_1617_2
16.3 0428-Pas5846_10603_2
17.5 0433-Pas6300_17861_2
20.4 0143-Pas293_67503_23
27.3 0060-Pas16602_3059_9
27.7 0048-Pas20321_1359_2
28.1 0183-Pas16145_1978_1
29.0 0305-Pas11060_7015_1
31.0 0368-Pas2231_19267_2
40.0 0202-Pas21_66815_207
42.1 0387-Pas26777_5417_8
42.8 0375-Pas2415_0_0_308
44.3 1032-pas1517_42369_1
46.1 0199-Pas19529_1502_1
46.9 0157-Pas10770_3675_1
47.3 0372-Pas2374_39785_5
51.4 0422-Pas542_30419_35
60.2 0004-Pas3342_67798_5
79.0 0121-Pas25183_3913_2
94.4 0165-Pas117_48699_26
94.9 0220-Pas2715_31086_9
103.9 1069-pas4513_0_0_209

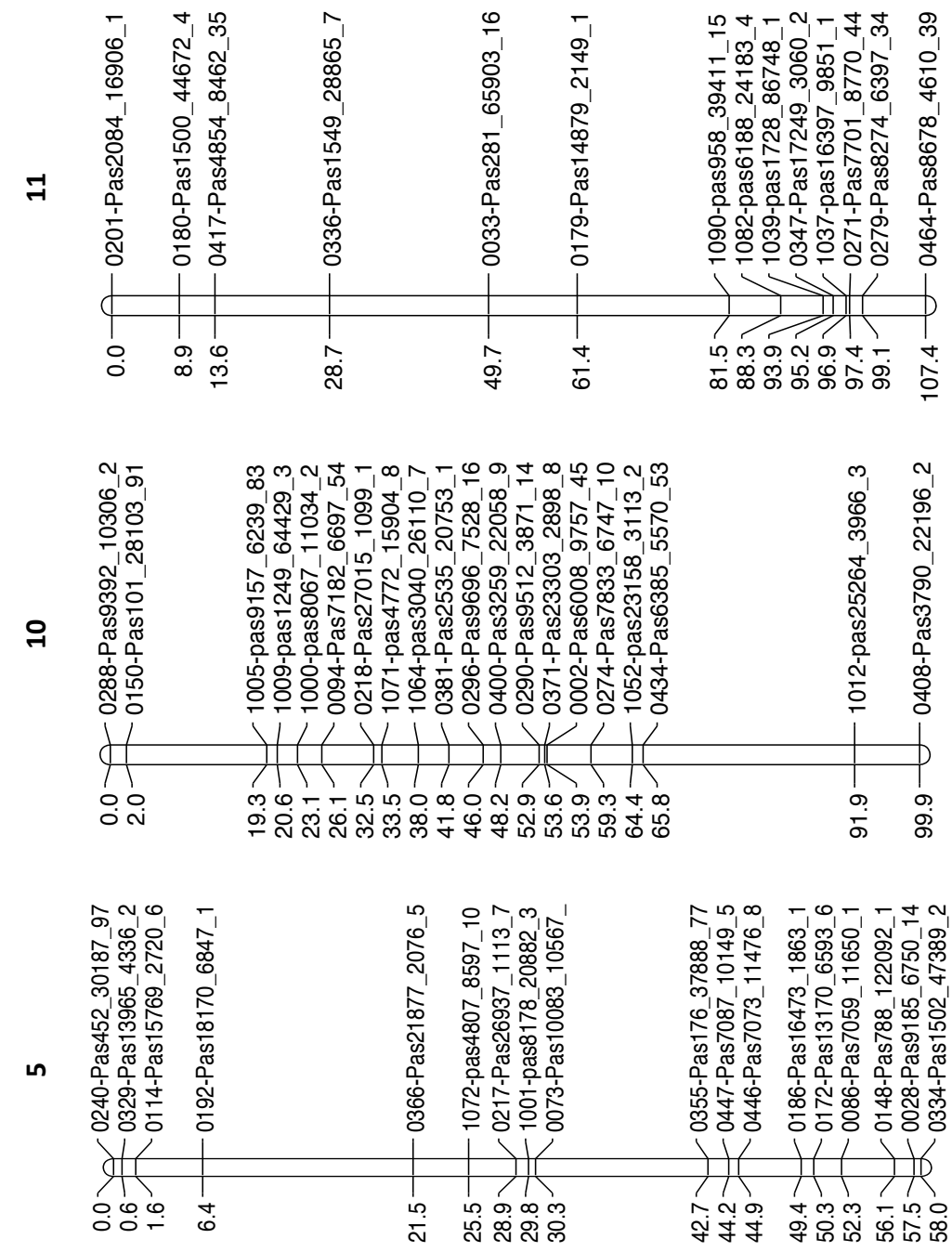


Figure 5.15 – SNP linkage map of *P. sativa* based on the P20 x P22 F_2 mapping population. Recombination distances are in cM. Numerical value refers to assigned linkage group number.

5.3.12 QTL analysis

The REML analyses of the phenotype trait data demonstrated a significant variation between lines for development of lesions, after inoculation with *I. pastinacae* isolate IP10 and *M. acerina* isolate MA2. The adjusted means from the REML analyses were combined with the linkage map to carry out QTL analysis on the five traits; *I. pastinacae* inoculated roots 2013/14, *I. pastinacae* inoculated roots 2014/15, *M. acerina* inoculated roots 2013/14, *M. acerina* inoculated roots 2014/15 and *M. acerina* inoculated roots 2015/16. The QTL analysis was performed separately on the data for each of the phenotype traits as described above. The analysis calculated the linkage group specific and genome wide 95% LOD significance thresholds (Table 5.12); these were used to infer QTLs.

Interval mapping was carried out in R/qtl, using a Haley-Knott regression. This process identified a QTL for the trait *M. acerina* 2014/15 (Fig 5.16), marker Pas1021_98532_5 on linkage group 6 at 32.24 cM with a LOD score of 3.56, just above the significance threshold of 3.51 (Table 5.12). Multiple-QTL mapping (MQM) through MapQTL was then used to enhance the sensitivity for this QTL using co-factors. A number of cofactors were examined and tested based upon the linkage to the trait on different chromosomes. One cofactor was found, Pas28073_1418_1 on linkage group 3, which improved the significance of the peak LOD score on linkage group 6 to a LOD value of 3.69. No other cofactors were found to further improve this value (Fig 5.17). The 95% LOD confidence interval for this QTL confers to Pas6193_8783_13 – Pas6550_2592_1. The increasing allele in this QTL was ‘a’, indicating the positive allele conferring to resistance is heritable from parent P20. The proportion of the total variation explained was 11.2%.

Table 5.12 – Linkage group and number of markers with linkage group specific and genome wide 95% LOD significance thresholds for P20 x P22 mapping population of 274 markers as determined using a permutation test.

		Linkage group gpecific 95% LOD significance threshold				
Linkage Group	Number of Markers	*I. pastinacae* 2013/14	*I. pastinacae* 201414/15	*M. acerina* 2013/14	*M. acerina* 2014/15	*M. acerina* 2015/16
1	33	0.482	1.681	1.795	0.493	1.671
2	28	0.927	1.101	2.328	0.764	1.505
3	32	0.242	1.820	1.257	1.955	1.659
4	30	1.621	0.986	0.783	0.303	1.516
5	22	2.624	0.895	2.287	0.532	1.317
6	32	0.989	1.014	0.783	3.561	2.221
7	21	0.791	0.315	1.651	0.764	1.079
8	24	1.119	0.930	0.907	1.286	0.886
9	18	0.566	1.046	0.656	2.322	1.510
10	20	1.659	1.018	2.602	1.022	0.498
11	14	0.834	0.487	2.037	0.504	1.285
Genome wide 95% LOD significance threshold		3.48	3.44	3.49	3.51	3.69

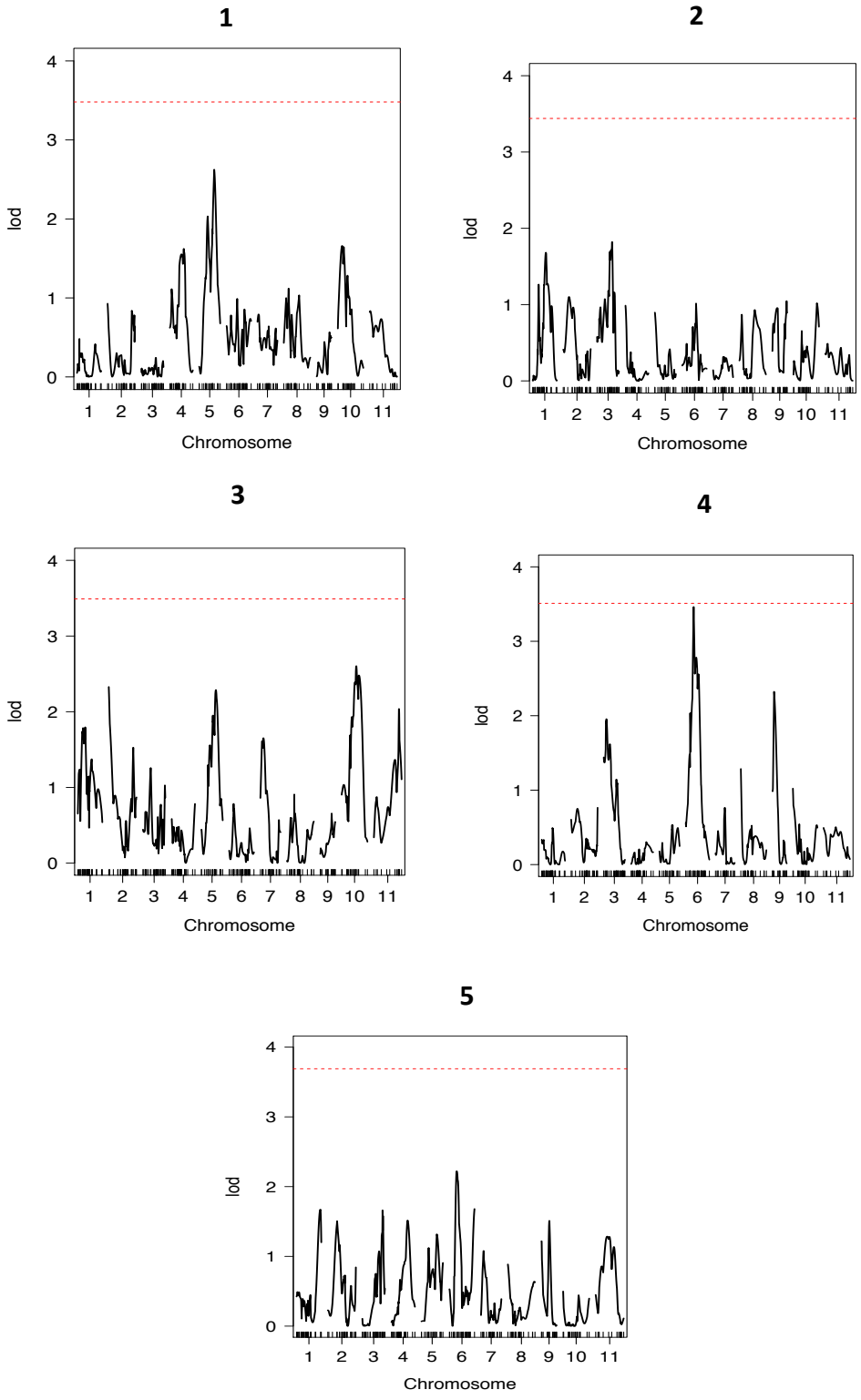


Figure 5.16 – LOD score graphs illustrating linkage results and 95% threshold for each of the traits. 1) *I. pastinacae* 2013/14. 2) *I. pastinacae* 2014/15. 3) *M. acerina* 2013/14. 4) *M. acerina* 2014/15. 5) *M. acerina* 2015/16.

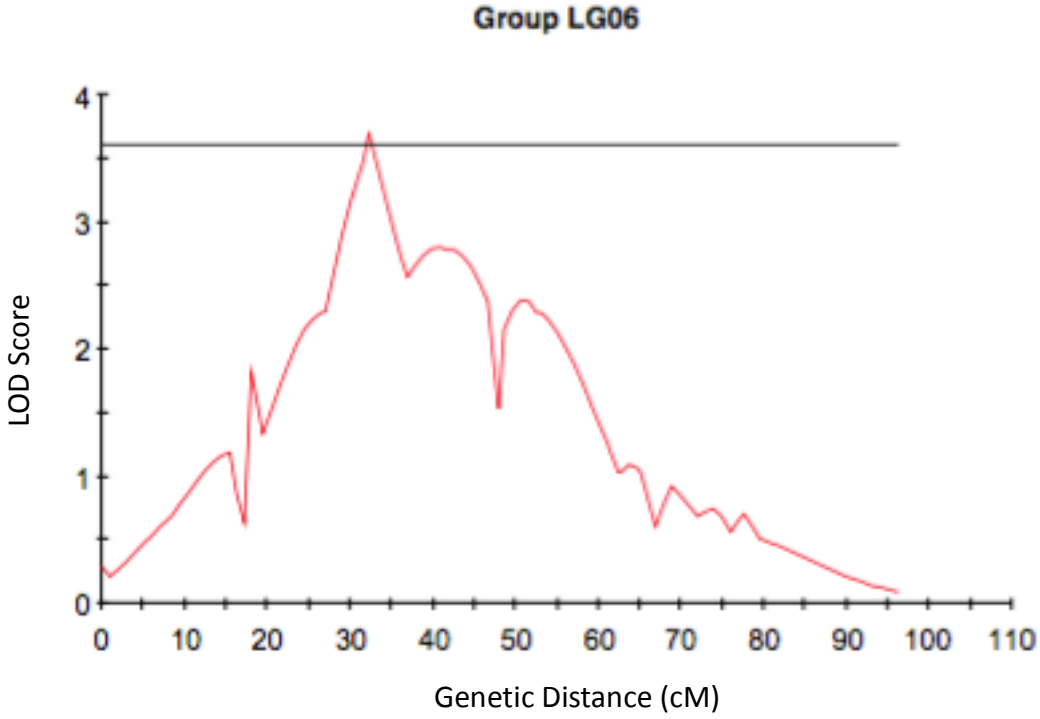


Figure 5.17 – LOD score and genetic distance (cM) for linkage group 6 of trait four (*M. acerina* inoculated parsnip roots 2014/15) with 95% LOD significance threshold.

5.4 Discussion

There is limited literature concerning breeding for disease resistance in parsnips, with the most recent work being that of Channon (1970). Commercial breeding companies are now looking to modern genetic technologies to improve the accuracy and speed of crop breeding programmes. Achieving this goal requires the development of a robust phenotyping assay, the identification of resistant lines and the subsequent mapping of resistance genes so that linked genetic markers can be identified for use in marker assisted selection. This work is an initial study into developing a large-scale phenotyping system to determine the degree of resistance within commercial parsnip breeding lines, with a SNP-genotyped parsnip mapping population also screened to identify QTLs conferring resistance. This research will have a direct impact on the industry by enabling the breeding of new parsnip varieties that are less susceptible to losses caused by root cankers.

The development of parsnip seedling and root assays to determine levels of resistance in response to *I. pastinacae*, *M. acerina* and *C. destructans* is novel. In addition, no previous research has identified seedling blight and taproot browning as symptoms of *I. pastinacae* infection. Furthermore, the work described here provides the first evidence of taproot browning symptoms as a response to *M. acerina* and *C. destructans* infection in parsnip seedlings. The development of these seedling assays offers a higher throughput alternative to root assays; waiting for roots to grow and reach a suitable size for inoculation mean large-scale phenotyping is time-consuming and not always practical in a commercial setting. In contrast, the seedling assays are quick, reproducible, and provide reliable results for identification of resistant lines.

In the *I. pastinacae* root and seedling assays, a range of resistance levels was observed between the parsnip parent lines. Significant differences between lines were observed in both experiments indicating some parsnip lines were more

resistant to *I. pastinacae* than others. There was little consistency between the results of the two assays with only Panache performing comparably between them. Many lines displayed contrasting results. For example, in the root assay line P15 displayed the smallest lesion area, and therefore the highest resistance, while in the seedling assay it displayed the highest percentage of seedling disease and was therefore the most susceptible. Similarly, P25 displayed a larger lesion area in the root assay and lower percentage seedling disease indicating this line was susceptible and resistant in the two assays respectively. This suggests different responses to resistance may be expressed at different stages of plant development.

In the *M. acerina* and *C. destructans* root and seedling resistance assays, again a range of resistance was observed between the parsnip parent lines, with significant differences between lines present in both experiments. A number of lines responded similarly in both assays: for example, lines P15 and P9 displayed small lesion areas and low percentage seedling disease in the *M. acerina* root and seedlings assay, respectively, whilst P22 and P23 were highly susceptible in both assays. A similar response was observed in the *C. destructans* root and seedling resistance assays, with lines P9, P14 and P23 displaying low resistance in both. Therefore, the only line to display similarly low levels of resistance to both pathogens across both assays was P23, while P9 displayed opposite levels of resistance to the two pathogens. This suggests resistance to both *M. acerina* and *C. destructans* is not conferred by the same loci.

When assessing results from both the root and seedling assays for all three pathogens, *I. pastinacae*, *M. acerina* and *C. destructans*, no obvious correlation was detected. For example, P23 displayed high levels of resistance to the first but low levels to the second and third, despite all three pathogens leading to comparable lesion areas in individual lines. This further confirms the hypothesis that resistance to root canker pathogens in parsnips is not conferred by a single gene, suggesting instead that different resistance mechanisms are employed by

the host in response to different pathogens. In addition, this work also highlights the importance of assessing resistance across various stages of plant development. This suggests resistance may be ontogenic, that is, varying levels of resistance can be displayed by a host in response to age or the tissue affected. This form of resistance can be found in response to a variety of pathogens on a range of plants. An example of this is powdery mildew on grapes, where grape berries are highly susceptible to powdery mildew until four weeks after fruit set, when they become nearly immune to infection (Ficke *et al.*, 2002). This has also been shown in edible burdock leaves following inoculation with *I. perplexans* where, as previously mentioned in Chapter 2, leaves younger than 5 days are highly susceptible to infection whileleaves older than 30 days are essentially immune (Horita & Yasuko, 2002).

The lines P20 and P22 were of particular interest in these experiments as they, are the parents of the cross used to make the genotyping population used for QTL mapping. This cross was selected by Elsoms Seeds Ltd prior to the start of this project, based on resistance experiments against a range of pathogens, in addition to the identification of other marketable traits. These experiments identified P20 as a line showing resistance and P22 as showing susceptibility to root canker pathogens. However, from the analysis conducted here, this is only visible for *M. acerina*, while in response to *I. pastinacae* and *C. destructans* P20 and P22 appear to perform equally in both assays. This suggests that the segregating population for phenotyping and mapping may be a more suitable approach for *M. acerina* than for the other two pathogens.

The resistance assay for the Elsoms breeding line population identified a range of significantly different levels of resistance across lines in response to both *M. acerina* and *I. pastinacae*. Little correlation was identified between the two pathogens, although white parsnip roots were identified as being highly susceptible to both pathogens. Specific consistencies were observed in line B21, which was highly susceptible in both assays, and B5, B8 and B26, which were

resistant to both pathogens. All parsnip line selections to date, including the breeding population, have been based on field selection. As shown in Chapter 2, a complex of fungal pathogens have been identified as causal agents for root canker disease, so selecting for specific resistance in a field setting can be difficult, leading to highly variable results. By developing these novel assays, it will be possible for commercial breeding programmes to select lines for resistance to a specific pathogen. Whilst traditional field selection provided good results for breeding consistent shape, yield and uniformity, breeding for resistance has been far more challenging.

Overall the results of the breeding population resistance assay indicate a range of resistance within commercial breeding lines. However, it has to be noted that these experiments were performed under controlled conditions and without the specific cropping guides of each line being taken into account. It is therefore possible that, when grown in a field situation, these lines may display more variable resistance responses.

Experiments to assess resistance in the P20 x P22 mapping population identified a range of resistance in response to both *I. pastinacae* and *M. acerina* over two and three years respectively. A significant difference was identified between the 139 lines, but like the previous resistance assays no correlation was detected across pathogens or years. The original genetic map of 433 KASP markers developed by Elsosms Seeds Ltd and Genetwister was based on genotyping the F_2 generation. However, as this did not include the F_2 parents of 64 of the phenotyped $F_{2:3}$ families, three $F_{2:3}$ individuals for each of these families were genotyped to enable the genotype of these F_2 parents to be inferred and incorporated into an updated version of the genetic map. The REML means for the $F_{2:3}$ families was used as the phenotypic input for QTL analysis and this was successful in identifying one significant QTL on linkage group 6 for the 2014/15 season *M. acerina* phenotype data. This QTL explained about 11% of the variance, which, given the usually large environmental component to the

variance (typically in the region of 50%), suggests that other loci may also contribute to the observed resistance. The fact that other QTL for this trait-year were not detected, or indeed not detected for the other traits/years, indicates that there was not enough power in the data to achieve this. This is likely due to the fact that different generations of the cross were genotyped and phenotyped. A large proportion of the phenotyped $F_{2:3}$ individuals would have been expected to be segregating for resistance alleles and hence provide additional noise in the data. One possibility to address this issue with the available data would be to rebuild the genetic map for the actual 192 (i.e. 3 x 64) genotyped $F_{2:3}$ individuals and use this to perform the QTL analysis. By doing this, the availability of the genotype of all phenotyped individuals might improve the power for QTL detection. However, the QTL that has been identified does now provide potentially useful linked markers that can be tested for their value for marker-assisted selection, which would overcome the phenotyping difficulties described above.

The increasing allele identified in the QTL conferring to resistance was heritable from parent P20. From the parsnip seedling and root resistance assays, P20 was identified as a resistant line in response to *M. acerina* inoculation. This suggests a QTL conferring resistance has been identified for use in marker assisted breeding.

6. General Discussion

Parsnip canker caused by fungal pathogens continues to be a major cause of crop losses in the UK. There have been limited studies examining the range of pathogens associated with root canker symptoms and identifying the primary pathogens responsible. The work of Channon (1963a, 1964, 1981) was seminal in identifying *I. pastinacae*, *M. acerina* and *C. destructans*, together with *Phoma* spp., as the primary causes of canker in parsnip roots. Since then, no other comprehensive studies have characterised the diversity of these primary pathogens in parsnips. Control measures for parsnip canker are limited, with no specific fungicides available and cultural practices only providing minor reductions in canker prevalence. As a result, there is a desire for alternative, long-term forms of control such as plant resistance. Traditional parsnip breeding can take up to 20 years to produce a new line. However, modern breeding in large acre crops now utilises the latest technologies, such as marker assisted breeding, to significantly increase the efficiency of this process. This project is extremely timely, as the new sequencing technologies allow us to apply such practices to parsnip. The aims of this research, as outlined in Chapter 1, were to characterise pathogens responsible for causing parsnip cankers and to develop tools to facilitate breeding for quantitative resistance to parsnip root canker diseases.

The results from the isolation and identification of pathogens responsible for parsnip canker, as described in Chapter 2, highlighted a range of pathogens associated with parsnip root blemishes. For the first time, this study showed parsnip canker symptoms to be clearly associated with certain pathogens, which in turn have been confirmed by molecular methods. This has therefore provided a more robust basis for identifying the causal agents of the different types of parsnip canker. *C. destructans*, *M. acerina* and, to a lesser extent, *I. pastinacae*

were identified as the main species responsible for causing parsnip canker, which provides important information commercially, for growers and breeders in designing more targeted control approaches and breeding programmes to provide more effective control of parsnip canker.

The *Itersonilia* spp. isolate characterisation represents the first study where *Itersonilia* spp. isolates from other hosts have been found to cause canker symptoms on parsnip. Following root and leaf inoculation experiments, all *Itersonilia* spp. isolates from a range of hosts, including parsnip, chrysanthemum and dill, were found to cause canker symptoms on parsnip roots and necrotic lesions on parsnip leaves. It is also the first study to quantify the effect of temperature on growth rate and spore production. These results confirm the ability of *Itersonilia* spp. to grow at low temperatures, a defining characteristic of similar species within the Cystofilobasidiales order. In addition, chlamydospore production in all *Itersonilia* spp. isolates was higher than ballistospores at low temperatures, with higher numbers of ballistospores produced at high temperatures. Both of these results are consistent with the hypothesised mode of transmission, suggesting high ballistospore numbers in summer, leading to necrotic foliar lesions. As the temperature drops, chlamydospore production increases with dying plant tissue falling into the soil, resulting in increased chlamaydospore inoculum in the soil. These chlamydospores are then the mode of infection for parsnip root cankers, or act as long-lived structures to ensure survival through to the next cropping season.

Itersonilia spp. is a dimorphic fungus, being isolated from hosts as a fungal pathogen but transitioning to a yeast phase after culturing. This study utilised transcriptome sequencing and gene expression analysis of both the yeast and fungal phase to identify up-regulated genes in the fungal phase, potentially relating to pathogenicity, and up-regulated genes in the yeast phase, possibly linked to the morphogenic transition from fungal to yeast. Additionally, whole genome sequencing enabled *Itersonilia* spp. specific primers to be designed for

the development of a rapid and reliable PCR diagnostic assay. This assay has the potential to improve accuracy and productivity in commercial seed testing. Although further experiments to determine the pathogenicity of *Itersonilia* spp. isolates on a range of hosts are required, the results observed here in the biological and molecular characterisation of isolates do not support the current distinction between *Itersonilia* spp., therefore indicating that *Itersonilia* should be described as a single species.

The characterisation of *C. destructans* and *M. acerina* isolates represents the first comprehensive analysis where multiple isolates from parsnips have been directly compared. Novel assays were designed for determining relative pathogenicity of the isolates on parsnip roots and seedlings. Experiments to determine the effect of temperature on mycelial growth found both *C. destructans* and *M. acerina* to grow at low temperatures, whilst sporulation experiments identified temperature to have limited effect on spore production in *C. destructans* isolates but the presence of UV light to lead to a significant increase in spore production in *M. acerina* isolates. This is also the first time *C. destructans* and *M. acerina* isolates from parsnips hosts have been characterised using molecular techniques. A range of diversity between isolates was identified, but clades were not delimited by host or pathogen species, therefore suggesting these two pathogens are part of a species complex that cannot be resolved by the ITS locus alone. Further multigene sequence data incorporating additional hosts, as well as the currently described anomorphs and teleomorphs, would be needed to provide further resolution for these phylogenetic analyses.

As the three main species associated with parsnip canker, the characterisation of *Itersonilia* spp., *C. destructans* and *M. acerina* provides valuable information for growers, breeders and the wider industry. All three pathogens were confirmed as being able to grow at low temperatures and produce chlamydospores, which are known to be able to survive from one cropping season to the next. These aspects are consistent with the high prevalence of parsnip canker observed by

growers during winter. However, the increased growth rate of all pathogens at high temperatures and the high spore production associated with summer conditions suggest these pathogens may be present in all seasons. Based on this information, developing targeted control approaches would be challenging. Consequently, the development of pathogen specific breeding programmes should provide a more robust and effective control strategy against parsnip canker.

As described above, due to the lack of specific control methods for parsnip canker, long-term sustainable resistance to these three pathogens is highly desirable. From the results of the *Itersonilia* spp., *M. acerina* and *C. destructans* isolate characterisation experiments, limited variation between isolates was observed. Therefore, a single representative isolates from each of these species was selected for use in breeding experiments to assess levels of resistance in parsnip lines. Novel parsnip root and seedling assays were developed in this study to determine levels of resistance within parsnip populations. The results indicated a range of resistance within parsnip populations in response to the three pathogens investigated. Through previous consultation with growers, Elsoms Seed Ltd focussed efforts on resistance to *I. pastinacae* and *M. acerina*. A segregating parsnip genotyping population derived from the parent line cross P20 x P22, was developed based on experiments identifying P20 as resistant and P22 as susceptible to root canker pathogens. However, from the current analysis, this appeared to be only true for *M. acerina*, while in response to *I. pastinacae* and *C. destructans* P20 and P22 appeared to perform equally in both assays.

In this study a range of phenotypes were observed in response to *I. pastinacae* and *M. acerina* root inoculation on parsnip lines from the P20 x P22 genotyping population. A significant QTL on linkage group 6 for the 2014/15 season *M. acerina* phenotype data was identified. The variance explained by this QTL suggests other loci may also contribute to the observed resistance; however, no other QTL were identified in this study. This is most likely due to different

generations of the cross being phenotyped and genotyped, where $F_{2:3}$ individuals would be more segregated for resistance alleles and therefore provide additional data. The increasing allele identified in the QTL conferring resistance to *M. acerina* was heritable from parent P20, which was observed in resistance assays as being highly resistant to *M. acerina* inoculation. This therefore suggests a QTL conferring resistance to *M. acerina* has been identified for use in marker-assisted breeding. By selecting different parent lines, a more informative segregating population could be developed to map resistance *for I. pastinacae* and *C. destructans*, as P20 and P22 did not segregate for resistance to these two pathogens.

Future work could follow different directions. First, this study designed robust methods for identifying the causal agents of parsnip canker through root isolations, and molecular identification. However, as chlamydospores are the primary source of root infection, there is a need to design a quantitative assay for assessing the concentrations of these primary fungal pathogens in the soil. Using the whole genome sequences of *I. pastinacae* and *M. acerina*, q-PCR assays could be developed to determine the specific levels of spores within the soil. In addition, given that for *I. pastinacae* ballistospores are implicated in the transmission of the pathogen from parsnip foliage to root, field spore trapping experiments combined with q-PCR assays could provide additional evidence to support the currently hypothesised mode of transmission.

Second, *Itersonilia* spp., *M. acerina* and *C. destructans* are all known to have a wide host range. However, there is currently limited evidence that isolates originating from one host could infect another, different host. In this study it was shown that *Itersonilia* spp. isolates from other hosts could cause canker-like symptoms on parsnip roots, but it remains unclear whether *Itersonilia* spp. isolates from parsnip could cause canker-like symptoms on other hosts, or whether similar cross-species behaviour holds also for *C. destructans* and *M.*

acerina isolates. For this, additional isolates of each pathogen from a range of hosts would be required, and a range of pathogenicity experiments designed.

Third, the gene expression analysis of *Itersonilia* spp. fungal and yeast phases identified up-regulated and down-regulated genes of interest that could relate to pathogenicity or the morphological transition between phases. Further analysis is required to ascertain whether any gene loss occurs in the fungus-to-yeast transition. Evidence of this would support the hypothesis of Ingold (1984) that such a transition is irreversible.

Finally, as previously described, the P20 x P22 cross did not segregate for resistance to *I. pastinacae* or *C. destructans*. By selecting different, more appropriate parent lines, a more accurate analysis can be carried out in order identify QTL conferring resistance to these pathogens, for implementation into a marker assisted breeding programme.

These future research directions would generate further understanding of the main causative agents of parsnip canker, with substantial benefit for growers, breeders and the general industry. Furthermore, the developed methods might be applicable to other pathogens and different hosts. Although substantial work is still needed for successful development of resistance to parsnip canker, the present research provides a comprehensive understanding of the causal agents of parsnip canker and for the first time applies modern sequencing technologies to develop a marker assisted breeding programme for resistance in parsnips.

References

Agrios, G.N., 2005. *Plant pathology*, Elsevier Academic Press Amsterdam, The Netherlands.

Alaniz, S. et al., 2007. Characterization of Cylindrocarpon species associated with black foot disease of grapevine in Spain. *Plant Disease*, **91**, 1187–1193.

Altschul, S.F. et al., 1990. Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Amarasinghe, C.C., Tittlemier, S.A. & Fernando, W.G.D., 2015. Nivalenol-producing *Fusarium cerealis* associated with fusarium head blight in winter wheat in Manitoba, Canada. *Plant Pathology*, **64**, 988–995.

Aquino-Piñero, E.E. & Rodríguez-del Valle, N., 1997. Different protein kinase C isoforms are present in the yeast and mycelium forms of *Sporothrix schenckii*. *Mycopathologia*, **138**, 109–115.

Aroca, A. & Raposo, R., 2007. PCR-based strategy to detect and identify species of Phaeoacremonium causing grapevine diseases. *Applied and Environmental Microbiology*, **73**, 2911–2918.

Arsvoll, K., 1969. Pathogens on carrots in Norway.

Averill, K.M. & DiTommaso, A., 2007. Intriguing World of Weeds Wild Parsnip (*Pastinaca sativa*): A Troublesome Species of Increasing Concern. *Weed Technology*, **21**, 279–287.

AY, R. et al., 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). *Studies in Mycology*, **42**, 1–260.

Ayerst, G., 1969. The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research*, **5**, 127–141.

Banihashemi, Z. & Dezeeuw, D.J., 1973. Saprophytic activities of *Fusarium oxysporum* f. sp. *melonis* in soil. *Transactions of the British Mycological Society*, **60**, 205–210.

Boekhout, T., 1948. Itersonilia Derx. *Descriptions of anamorphic basidiomycetous genera and species*, 775–776.

Boekhout, T., 1991a. Systematics of Itersonilia: a comparative phenetic study. *Mycological Research*, **95**, 135–146.

Boekhout, T. et al., 1991b. Genomic characteristics of strains of Itersonilia: taxonomic consequences and life cycle. *Canadian Journal of Microbiology*, **37**, 188–194.

Boland, G.J. & Hall, R., 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology*, **16**, 93–108.

Bölker, M, 2001. Ustilago maydis – a valuable model system for the study of fungal dimorphism and virulence. *Microbiology*, 147, 1395-1401.

Braun, P.G., 1995. Effects of Cylindrocarpon and Pythium species on apple seedlings and potential role in apple replant disease. *Canadian Journal of Plant Pathology*, **17**, 336–341.

Brayford D., 1992. *Cylindrocarpon. In: Methods for Research on Soilborne Phytopathogenic Fungi.* L. L. Singleton, J. D. Mihail, & C. M. Rush, eds., St Paul, MN, USA: American Phytophathological Society.

Broman, K.W. et al., 2003. R/qtl: QTL mapping in experimental crosses. *Bioinformatics*, **19**, 889–890.

Brown, A., Nourish, W.H. & Fletcher, J.T., 1964. The effect of spacing and time of sowing on the incidence of parsnip canker. *Journal of Horticultural Science*, **39**, 103–110.

Byrnes, E.J. et al., 2011. *Cryptococcus gattii*: an emerging fungal pathogen infecting humans and animals. *Microbes and Infection*, **13**, 895–907.

Cabral, A. et al., 2012. Cylindrocarpon root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicicola* species complex. *Mycological Progress*, **11**, 655–688.

Cain, N. et al., 2010. The Biology of Canadian weeds . 144 . *Pastinaca sativa* L . *Canadian Journal of Plant Science*, **90**, 217–240.

Castlebury, L.A., Rossman, A.Y. & Hyten, A.S., 2006. Phylogenetic relationships of Neonectria/Cylindrocarpon on Fagus in North America. *Botany*, **84**, 1417–1433.

Cerkauskas, R.F., 1987. Phoma canker severity and yield losses in parsnip. *Canadian Journal of Plant Pathology*, **9**, 311–318.

Cerkauskas, R.F., 1985. Canker of parsnip caused by *Phoma complanata*. *Canadian Journal of Plant Pathology*, **7**, 135–138.

Channon, A.G., 1965. Studies on parsnip canker IV. *Centrospora acerina* (Hartig) Newhall - a further cause of black canker. *Annals of Applied Biology*, **56**, 119–128.

Channon, A.G., 1963a. Studies on Parsnip canker I. The causes of the diseases. *Annals of Applied Biology*, **51**, 1–15.

Channon, A.G., 1963b. Studies on parsnip canker II. Observations on the occurrence of *Itersonilia pastinacae* and related fungi on the leaves of parsnips and in the air within parsnip crops. *Annals of Applied Biology*, **51**, 223–230.

Channon, A.G., 1964. Studies on parsnip canker III. The effect of sowing date and spacing on canker development. *Annals of Applied Biology*, **54**, 63–70.

Channon, A.G., 1969. Infection of the flowers and seed of parsnip by *Itersonilia pastinacae*. *Annals of Applied Biology*, **64**, 281–288.

Channon, A.G., Dowker, B.D. & Holland, H., 1970. The breeding of Avonresister, a canker-resistant parsnip. *Journal of Horticultural Science*, **45**, 249–256.

Channon, A.G. & Thomson, M.C., 1981. New or unusual records of plant diseases and pests - II. Parsnip canker caused by Cylindrocarpon destructans. *Plant Pathology*, **30**, 181.

Coleman, D.C., 2008. From peds to paradoxes: Linkages between soil biota and their influences on ecological processes. *Soil Biology & Biochemistry*, **40**, 271–289.

Collard, B.C.Y. et al., 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. *Euphytica*, **142**, 169–196.

Collard, B.C.Y. & Mackill, D.J., 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**, 557–572.

Collingwood, C.A. & Croxall, H.E., 1948. Carrot fly injury and 'canker' of parsnips in the West Midlands. *Plant Pathology*, **3**, 99–103.

Conners, I.L., 1967. *An annotated index of plant diseases in Canada.*, Ottawa, ON: Researhc Branch - Canda Department of Agriculture.

Correll, J., 1991. The relationship between formae speciales, races, and vegetative compatibility groups in *Fusarium oxysporum*. *Phytopathology*, **81**, 1061–1064.

Crous, P.W. et al., 2009. Phylogeny and taxonomy of obscure genera of microfungi. *Persoonia*, **22**, 139–61.

Crous, P.W. et al., 2009. Phylogenetic lineages in the Capnodiales. *Studies in Mycology*, **64**,17–47.

Davidson, G. et al., 2003. Study of temperature–growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development. *Journal of Applied Microbiology*, **94**, 816–825.

Davies, W.P., 1977. Infection of carrot roots in cool storage by *Centrospora acerina*. *Annals of Applied Biology*, **85**, 163–164.

Davies, W.P. & Lewis, B.G., 1981. Antifungal activity in carrot roots in relation to storage infection by *Mycocentrospora acerina* (Hartig) Deighton. *New Phytologist*, **89**, 109–119.

Day, J.R., Lewis, B.G. & Martin, S., 1972. Infection of stored celery plants by *Centrospora acerina*. *Annals of Applied Biology*, **71**, 201–210.

DeVoti, J. et al., 1991. Interaction between ran1+ protein kinase and cAMP dependent protein kinase as negative regulators of fission yeast meiosis. *The EMBO Journal*, **10**, 3759.

Dirmenci, T., 2008. A new species of Pastinaca L . (Apiaceae) from Turkey. *Botanical Journal of the Linnean Society*, **158**, 296–300.

Dorn-In, S. et al., 2013. PCR-SSCP-based reconstruction of the original fungal flora of heat-processed meat products. *International Journal of Food Microbiology*, **162**, 71–81.

Dosdall, L.T., 1956. A petal blight of Chrysanthemum incited by *Itersonilia perplexans*. *Phytopathology*, **46**, 231–232.

Dumroese, R.K., James, R.L. & Wenny, D.L., 2000. An Assessment of Cylindrocarpon on Container Western White Pine Seedlings After Outplanting. *Western Journal of Applied Forestry*, **15**, 5–7.

Dupont, S. et al., 2012. Ergosterol biosynthesis: A fungal pathway for life on land? *Evolution*, **66**, 2961–2968.

Edel, V. et al., 1997. Populations of Nonpathogenic *Fusarium oxysporum* Associated with Roots of Four Plant Species Compared to Soilborne Populations. *Phytopathology*, **87**, 693–697.

Elad, Y. et al., 2007. Botrytis spp. and diseases they cause in agricultural systems–an introduction. In *Botrytis: Biology, pathology and control*. Springer, pp. 1–8.

Engelberth, J., 2006. *Ch 13. Secondary Metabolites and Plant Defence, Plant Physiology* 4th ed. L. Taiz & E. Aeiger, eds., Sunderland, MA, USA: Sinauer Associates, Inc,.

Evenhuis, A., 1995. *On the ecology and control of Mycocentrospora acerina in caraway (Carum carvi)*.

Fell, J.W., Roeijmans, H. & Boekhout, T., 1999. Cystofilobasidiales, a new order of basidiomycetous yeasts. *International Journal of Systematic and Evolutionary Microbiology*, **49**, 907–913.

Ficke, A., Gadoury, D.M. & Seem, R.C., 2002. New Frontiers in Plant Disease Losses and Disease Management Ontogenic Resistance and Plant Disease Management : A Case Study of Grape Powdery Mildew. , **92**, 671–675.

Field, C.L. et al., 2012. Systemic Mycosis in a California Sea Lion (*Zalophus californianus)* with Detection of Cystofilobasidiales DNA. *Journal of Zoo and Wildlife Medicine*, **43**, 144–152.

Förster, H. & Mendgen, K., 1987. Immunocytochemical localization of pectinesterases in hyphae of *Phytophthora infestans*. *Canadian Journal of Botany*, **65**, 2607–2613.

Fox, R.T. V. & Narra, H.P., 2006. *Plant disease diagnosis. In The epidemiology of plant diseases.* 2nd EDITION. B. Eds. Cooke, B.M., Jones, D.G. and Kaye, E.

Francia, E. et al., 2005. Marker assisted selection in crop plants. *Plant Cell, Tissue and Organ Culture*, **82**, 317–342.

Gandy, D.G., 1966. *Itersonilia perplexans* on chrysanthemums: Alternative hosts and ways of overwintering. *Transactions of the British Mycological Society*, **49**, 499–507.

Garcia-Pedrajas, M., Baeza-Montañes, L. and Gold, S., 2010. Regulation of Ustilago maydis dimorphism, sporulation, and pathogenic development by a transcription factor with a highly conserved APSES domain. *Molecular plant-microbe interactions*, 23, 211-222.s

Garrido, L.R. & O.R. Sônego, A.F., 2004. Urben *Cylindrocarpon destructans* causador do 'Pé-Preto' da videira no Rio Grande do Sul. *Fitopatologia Brasileira*, **29**, 548–550.

Gill, D.L., 1971. *Centrospora acerina* carried by Pansy seed. *Plant Disease Reporter*, **55**, 731–732.

Gladders, P., 1997. *Parsnip: Control of Canker*, AHDB Final Report, FV366

Gleason, H.A. & Cronquist, A., 1991. *Manual of vascular plants of northeastern North America and adjacent Canada.*,

Goswami, R.S. & Kistler, H.C., 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology*, **5**, 515–525.

Grandillo, S., Tanksley, S.D. & Zamir, D., 2007. *Ch.6. Exploitation of Naturla biodiversity through genomics. Genomics assisted crop improvement Vol 1. Genomics Approaches and Platforms.* R. K. Varshney & R. Tuberosa, Eds., Dordrecht, The Netherlands: Springer.

Grieve, M., 1931. *A modern herbal. The medicinal, culinary, cosmetic and economic properties, cultivation and folk-lore of herbs, grasses, fungi, shrubs & trees with all their modern scientific uses.* XVI., Toronto, ON: Jonathon Cape.

Gupta P. k., Rustgi, S and Mir R. R., 2008. Array-based high-throughout DNA markers for crop improvement. *Heredity*, 101, 5-18.

Halleen, F., Fourie, P.H. & Crous, P.W., 2006. A Review of Black Foot Disease of Grapevine. *Phytopathologia Mediterranea*, **45**, 55–67.

Hawkes, J.G. & Francisco-Ortega, J., 1993. The early history of the potato in Europe. *Euphytica*, **70**, 1–7.

He, J. et al., 2014. Genotyping-by-sequencing (GBS), an ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. *Frontiers in Plant Science*, **5**, 484.

Hedrick, U.P., 1919. *Sturtevant's Notes on Edible Plants*, Albany, NY: J. B. Lyon.

Hendrix, S.D., 1984. Variation in Seed Weight and Its Effects on Germination in *Pastinaca sativa* L. (Umbelliferae). *American Journal of Botany*, **71**, 795.

Hendrix, S.D. et al., 1991. Are seedlings from small seeds always inferior to seedlings from large seeds? Effects of seed biomass on seedling growth in *Pastinaca sativa* L. *New Phytologist*, **119**, 299–305.

Hermansen, A. et al., 1997. *Mycocentrospora acerina* in carrots.pdf. *Annals of Applied Biology*, **131**, 399–411.

Hermansen, A. 1992. Weeds as hosts of *Mycocentrospora acerina*. *Annals of Applied Biology*, **121**, 679–686.

Hicks, R.G.T., Smith, T.J. & Edwards, R.P., 1986. Effects of strawberry latent ringspot virus on the development of seeds and seedlings of *Chenopodium quinoa* and *Pastinaca sativa* (parsnip). *Seed and Science Technology*, **14**, 409–417.

Horita, H. et al., 2005. Effects of inoculum density, leaf age, moisture, temperature, and wetness duration on black streak of edible burdock. *Journal of General Plant Pathology*, **71**, 247–252.

Horita, H. & Yasouka, S., 2002. Black streak ofedible burdock caused by *Itersonilia perplexans* in Japan. *The phytopathological Society of Japan*, **68**, 277–283.

Horsfall, J.G. & Dimond, A.E., 1960. *Plant Pathology: an advance treatise, volume II.*, New York, USA.: Academic Press.

Ingold, C.T., 1986. Chlamydospore ontogeny in Itersonilia. *Transactions of the British Mycological Society*, **86**, 501–503.

Ingold, C.T., 1983. Structure and development in an isolate of *Itersonilia perplexans*. *Transactions of the British Mycological Society*, **80**, 365–368.

Ingold, C.T., 1984. Further observations on Itersonilia. *Transactions of the British Mycological Society*, **83**, 166–174.

Iqbal, S.H. & Webster, J., 1969. Pathogenicity of aquatic isolates of *Centrospora acerina* to carrots and parsnips. *Transactions of the British Mycological Society*, **53**, 486–495.

James, R.L., 2008. Container Western White Pine Seedlings : Root Colonization by Fusarium and Cylindrocarpon Species. *Tree Planter's Notes*, **53**, 23–28.

Jansen, R.C., 1993. Interval mapping of multiple quantitative trait loci. *Genetics*, **135**, 205–211.

Jansen, R.C. & Stam, P., 1994. High resolution of quantitative traits into multiple loci via interval mapping. *Genetics*, **136**, 1447–1455.

Jones, P.E., 2001. *The development of diagnostic tools for the grapevine pathogen Eutypa lata: a thesis presented in partial fulfilment of the requirements for the degree of Master of Science*.

Kahmann, R., and Bölker, M., 1996. Self/nonself recognition in fungi: old mysteries and simple solutions. *Cell*, 85, 145-148.

Kartesz, J.T., 1994. *A synonymized checklist of the vascular flora of the United States, Canada and Greenland.*, Portland, OR: Timber Press.

Koike, S.T. & Tjosvold, S.A., 1991. A Blight Disease of Dill in California Caused by *Itersonilia perplexans*. *The American Phytopathological Society*, **85**, 802.

Kovalchuk, A., Lee, Y.-H. & Asiegbu, F.O., 2013. Diversity and evolution of ABC proteins in basidiomycetes. *Mycologia*, **105**, 1456–1470.

Kumpatla, S.P. et al., 2012. *Genomics-assisted plant breeding in the 21st century: technological advances and progress*, INTECH Open Access Publisher.

Lambourne, C., 2011. *Herbs: A survey into the prevalence and severity of Itersonilia* spp*. in UK crops*. AHDB Final Report 381a.

Lateef, D. D., 2015. DNA marker technologies in plants and applications for crop improvements. *Journal of Biosciences and Medicines*, 3, 7-18.

Laws, B., 2004. *Spade, Skirret and Parsnip: The curious history of vegetables.*, Stroud, United Kingdom: Sutton Publishing.

Leoni, C. et al., 2013. *Fusarium oxysporum* f.sp. *cepae* dynamics: In- plant multiplication and crop sequence simulations. *European Journal of Plant Pathology*, **137**, 545–561.

Loftus, B.J. et al., 2005. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science*, **307**, 1321–1324.

Lombard, L. et al., 2015. Generic concepts in Nectriaceae. *Studies in Mycology*, **80**, 189–245.

Loto, I. et al., 2012. Enhancement of carotenoid production by disrupting the C22-sterol desaturase gene (CYP61) in *Xanthophyllomyces dendrorhous*. *BMC Microbiology*, **12**, 235.

Mabberley, D.J., 1997. *The plant-book: a portable dictionary of the vascular plants*, Cambridge: Cambridge University Press.

Mackill, D.J. & Ni, J.J., 2001. Molecular mapping and marker-assisted selection for major-gene traits in rice. *Rice Genetics IV*, 140–141.

Madhani, H.D. & Fink, G.R., 1998. The control of filamentous differentiation and virulence in fungi. *Trends in Cell Biology*, **8**, 348–353.

Maluta, D.R. & Larignon, P., 1991. Pied-noir: Mieux vaut prevenir. *Viticulture*, **11**, 71–72.

Mammadov, J. et al., 2012. 'SNP Markers and Their Impact on Plant Breeding'. *International Journal of Plant Genomics*, **2012**, 11 pages.

Mantiri, F.R. et al., 2001. Phylogenetic relationships in Neonectria species having Cylindrocarpon anamorphs inferred from mitochondrial ribosomal DNA sequences. *Canadian Journal of Botany*, **79**, 334–340.

Margesin, R. & Fell, J.W., 2008. *Mrakiella cryoconiti* gen. nov., sp. nov., a psychrophilic, anamorphic, basidiomycetous yeast from alpine and arctic habitats. *International Journal of systematic and Evolutionary Microbiology*, **58**, 2977–2982.

Matejić, J.S. et al., 2014. Antimicrobial potential of essential oil from *Pastinaca sativa* L . *Biologica Nyssana*, **5**, 31–35.

Matturi, S.T. & Stenton, H., 1964. Distribution and status in the soil of Cylindrocarpon species. *Transactions of the British Mycological Society*, **47**, 577–587.

McGee, H., 1984. *On food and Cooking. The science and lore of the kitchen.*, New York: Charles Scribner's.

McGovern, R.J. et al., 2006. Host Range of *Itersonilia perplexans* and Management of Itersonilia Petal Blight of China Aster. *Plant Health Progress*, 1–9.

McPherson, G.M., 2013. *Parsnip: An improved understanding of root blemishes and their prevention (FV 366)*,

Millanes, A.M. et al., 2011. Phylogeny and character evolution in the jelly fungi (Tremellomycetes, Basidiomycota, Fungi). *Molecular Phylogenetics and Evolution*, **61**, 12–28.

Minchinton, E., 2008. *Identification of the extent and cause of parsnip canker (VG05045)*,

Ming-xiu, X.I.N. & Pei-jin, Z., 2007. *Mrakia psychrophila* sp. nov., a new species isolated from Antarctic soil. *J Zheijang Univ Sci B*, **8**, 260–265.

Ministry of agriculture, food and rural affairs, 2012. *C. destructans* infected ginseng (*Panax quinquefolium*) root, orange/brown corky lesion. Available at: http://www.omafra.gov.on.ca/CropOp/en/herbs/medicinal/gins.html.

Mir, R. R. *et al*., 2013. Evolving molecular marker technoliges in plants: From RFLPs to GBS. In: Lübberstedt, T. and Varshney, R. K., Eds., *Diagnostics in Plant Breeding*, Springer, Berlin, 229-247.

Mochizuki, N. & Yamamoto, M., 1992. Reduction in the intracellular cAMP level triggers initiation of sexual development in fission yeast. *Molecular and General Genetics MGG*, **233**, 17–24.

Mohler, V. & Singrün, C., 2004. General considerations: marker-assisted selection. In *Molecular Marker Systems in Plant Breeding and Crop Improvement*. Springer, pp. 305–317.

Morris, M.J. & Crous, P.W., 1994. New and Interesting records of South African fungi. XIV. Cercosporoid fungi from weeds. *South African Journal of Botany*, **60**, 325.

Nadal, M., García-Pedrajas, M.D. & Gold, S.E., 2008. Dimorphism in fungal plant pathogens. *FEMS Microbiology Letters*, **284**, 127–134.

Neergaard, P. & Newhall, A.G., 1951. Notes on the physiology and pathogenicity of *Centrospora acerina* (Hartig) Newhall. *Phytopathology*, **41**, 1021–1033.

Neiman, A.M. et al., 1993. Functional homology of protein kinases required for sexual differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* suggests a conserved signal transduction module in eukaryotic organisms. *Molecular Biology of the Cell*, **4**, 107–120.

Newhall, A.G., 1944. *A serious storage rot of celery caused by the fungus* Ansatospora macrospora *n. gen*.

Nyland, G., 1949. Studies on Some Unusual Hetero- basidiomycetes from Washington State. *Mycologia*, **41**, 688–701.

Olive, L.S., 1952. Studies on the Morphology and Cytology of *Itersonilia perplexans* Derx. *Bulletin of the Torrey Botanical Club*, **79**, 126–138.

Oliveira, H., Rego, M.C. & Nascimento, T., 2003. Decline of young grapevines caused by fungi. In *I International Symposium on Grapevine Growing, Commerce and Research* **652.** 295–304.

Orlowski, M. & Ross, J.F., 1981. Relationship of internal cyclic AMP levels, rates of protein synthesis and mucor dimorphism. *Archives of Microbiology*, **129**, 353–356.

Ostertag, E. et al., 2002. Effects of Storage Conditions on Furocoumarin Levels in Intact, Chopped, or Homogenized Parsnips. *Journal of Agricultural and Food Chemistry*, **50**, 2565–2570.

Pan, X., Harashima, T. & Heitman, J., 2000. Signal transduction cascades regulating pseudohyphal differentiation of Saccharomyces cerevisiae. *Current Opinion in Microbiology*, **3**, 567–572.

Paterson, A.H., Sorrells, M.E. & Obendorf, R.L., 1989. Methods of evaluation for preharvest sprouting resistance in wheat breeding programs. *Canadian Journal of Plant Science*, **69**, 681–689.

Perlin, M.H., Andrews, J. & Toh, S.S., 2014. *Essential Letters in the Fungal Alphabet: ABC and MFS Transporters and Their Roles in Survival and Pathogenicity. In Advances in Genetics: Vol. 35* T. Friedmann, J. Dunlap, & S. Goodwin, eds., Academic Press, Elsevier Inc.

Petit, E. & Gubler, W.D., 2005. Characterization of Cylindrocarpon species, the cause of black foot disease of grapevine in California. *Plant Disease*, **89**, 1051–1059.

Petkowski, J.E. et al., 2010. Fungi and oomycetes assicuated with root rot complex in parsnip crops. In *Proceedings of the 6th Australasian Soilborne Diseases Symposium*. 73.

Petkowski, J.E. et al., 2013. Pythium species associated with root rot complex in winter-grown parsnip and parsley crops in south eastern Australia. *Australasian Plant Pathology*, **42**, 403–411.

Pollack, F.G., 1987. *An annotated compilation of Cercospora names.*, J. Cramer.

Pringle, A. et al., 2005. The captured launch of a ballistospore. *Mycologia*, **97**, 866–871.

Probst, C.M., 2011. *Cylindrocarpon Black Foot Disease in Grapevines Identification and Epidemiology*.

Rego, C., Nascimento, T. & Oliveira, H., 2001. Characterisation of *Cylindrocarpon destructans* isolates from grapevines in Portugal. *Phytopathologia Mediterranea*, **40**, 343–350.

Rogers, S.L., Atkins, S.D. & West, J.S., 2009. Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathology*, **58**, 324–331.

Romberg, M.K. & Davis, R.M., 2007. Host range and phylogeny of *Fusarium solani* f. sp. *eumartii* from potato and tomato in California. *Plant Disease*, **91**, 585–592.

Rubatzky, V.E., 1997. *World Vegetables, Principal, Production & Nutritive Values.*, New York: Chapman & HAll, Internaltional Thompson Publishing.

Sackston, W.E., 1958. *Itersonilia perplexans* on sunflowers in Uruguay. *Phytopathology*, **48**, 108–109.

Samuels, G.J. & Brayford, D., 1990. Variation in *Nectria radicicola* and its anamorph, *Cylindrocarpon destructans*. *Mycological Research*, **94**, 433–442.

Samuels, G.J. et al., 2009. Cyanonectria , a new genus for *Nectria cyanostoma* and its Fusarium anamorph. *Mycological Progress*, **8**, 49–58.

Scheck, H. et al., 1998. Grape growers report lesses to black-foot and grapevine decline. *California Agriculture*, **8**, 19–23.

Schoolfield, R.M., Sharpe, P.J.H. & Magnuson, C.E., 1981. Non-linear regression of biological temperature-dependent rate models based on absolute reaction-rate theory. *Journal of Theoretical Biology*, **88**, 719–731.

Schwartz, M.A. & Madhani, H.D., 2004. Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. *Annual Review of Genetics.*,**38**, 725–748.

Seifert, K.A. et al., 2003. Molecular Differentiation and Detection of Ginseng-Adapted Isolates of the Root Rot Fungus *Cylindrocarpon destructans*. *Population Biology*, **93**, 1533–1542.

Seifert, K.A. et al., 2003. Molecular Differentiation and Detection of Ginseng-Adapted Isolates of the Root Rot Fungus *Cylindrocarpon destructans*. *Population Biology*, **93**, 1533–1542.

Semagn, K. *et al*., 2013. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. *Molecular Breeding*, 33, 1-14.

Sharpe, P.J.H. & DeMichele, D.W., 1977. Reaction kinetics of poikilotherm development. *Journal of Theoretical Biology*, **64**, 649–670.

Shattuck, V.I., Kakuda, Y. & Yada, R., 1989. Sweetening of Parsnip Roots During Short-Term Cold Storage. *Canadian Institute of Food Science and Technology*, **22**, 378–382.

Shin, H. & Kim, J., 2001. *Cercospora and allied genera from Korea*, National Institute of Agricultural Science and Technology.

Simmonds, N.W., 1976. *Evolution of crop plants.*, London & New York: Longman.

Sivasubramaniam, W., Wratten, S.D. & Frampton, C.M., 1999. Modifying the location and application rate of insecticides for carrot rust fly (*Psila rosae* F.) control in New Zealand. *International Journal of Pest Management*, **45**, 161–166.

Smith, P.R., 1966. Seed transmission of *Itersonilia pastinacae* in parsnip and its elimination by a steam-air treatment. *Australian Journal of Experimental Agriculture*, **6**, 441–444.

Society, R.H., 2016. M. acerina infected wild pansy (*Viola tricolor*); foliar symptoms include black lesions. Available at: https://www.rhs.org.uk/advice/profile?PID=571.

Sowell, G. & Korf, R.P., 1960. An Emendation of the Genus Itersonilia Based on Studies of Morphology and Pathogenicity. *Mycologia*, **52**, 934.

Stam, P. & Ooijen, J.W., 1995. *JoinMap: Version 2.0: Software for the Calculation of Genetic Linkage Maps*, CPRO-DLO.

Stewart, E.L. et al., 1999. Phylogenetic relationships among some cercosporoid anamorphs of Mycosphaerella based on rDNA sequence analysis. *Mycological Research*, **103**, 1491–1499.

Stone, L.E.W., 1954. Carrot fly and 'canker' of parsnips in the South-West. *Plant Pathology*, **3**, 118.

Surhone, L.M., Tennoe, M.T. & Henssonow, S.F., 2010. *Parsnip*, Beau Bassin, Mauritius: Betascript Publishing.

Sutton, B.C. & Gibson, I.A.S., 1977. *Mycocentrospora acerina*. *CMI Descriptions of pathogenic fungi and bacteria*, **357**.

Tamura, K. et al., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 3**0**, 2725–2729.

Tanksley, S.D., 1993. QTL analysis of transgressive segregation in an interspecific tomato cross. *Genetics*, **134**, 585–596.

Taylor, G.S., 1964. *Fusarium oxysporum* and *Cylindrocarpon radicicola* in relation to their association with plant roots. *Transactions of the British Mycological Society*, **47**, 381-391.

Tompkins, C.M. & Hansen, H.N., 1950. Pansy leafspot, caused by *Centrospora acerina*, host range, and control. *Hilgardia*, **19**, 383–389.

Trager, J., 1970. *the Food Book*, New York: Avon Books.

Tubaki, K., 1952. Studies on the sporobloromycetaceae in Japan. I. On Itersonilia. *Nagaoa*, **2**, 62–66.

Úrbez-Torres, J.R., Peduto, F. & Gubler, W.D., 2014. First report of *Ilyonectria macrodidyma* causing root rot of olive trees (*Olea europaea*) in California. *Plant Disease*, **98**, 456–468.

Van Ooijen, J.W., 2006. JoinMap 4 Software for the calculation of genetic linkage maps in experimental populations.

Wall, C.J. & Lewis, B.G., 1978. Quantitative studies on survival of *Mycocentrospora acerina* conidia in soil. *Transactions of the British Mycological Society*, **73**, 143–146.

Wall, C.J. & Lewis, B.G., 1980. Survival of chlamydospores and subsequent development of *Mycocentrospora acerina* in soil. *Transactions of the British Mycological Society*, **75**, 207–211.

Webster, J. et al., 1984. Ballistospore discharge in *Itersonilia perplexans*. *Transactions of the British Mycological Society*, **82**, 13–29.

Westerdijk, J. & VanLuijk, A., 1924. Eine Anthraknose des Kümmels (Carumcarvi). *Mededelingen Phytopathologisch Laboratorium 'WCS'*, **8**, 51–54.

Wheatley, G.A. & Freeman, G.H., 1982. A method of using the proportions of undamaged carrots or parsnips to estimate the relative population densities of carrot fly (*Psila rosae*) larvae, and its practical applicatins. *Annals of Applied Biology*, **100**, 229–244.

White, T.J. et al., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, **18**, 315–322.

Wilkinson, R.E., 1952. Parsnip canker is caused by Itersonilia sp. *Phytopathology*, **42**, 23.

Witcombe, J.R. & Virk, D.S., 2001. Number of crosses and population size for participatory and classical plant breeding. *Euphytica*, **122**, 451–462.

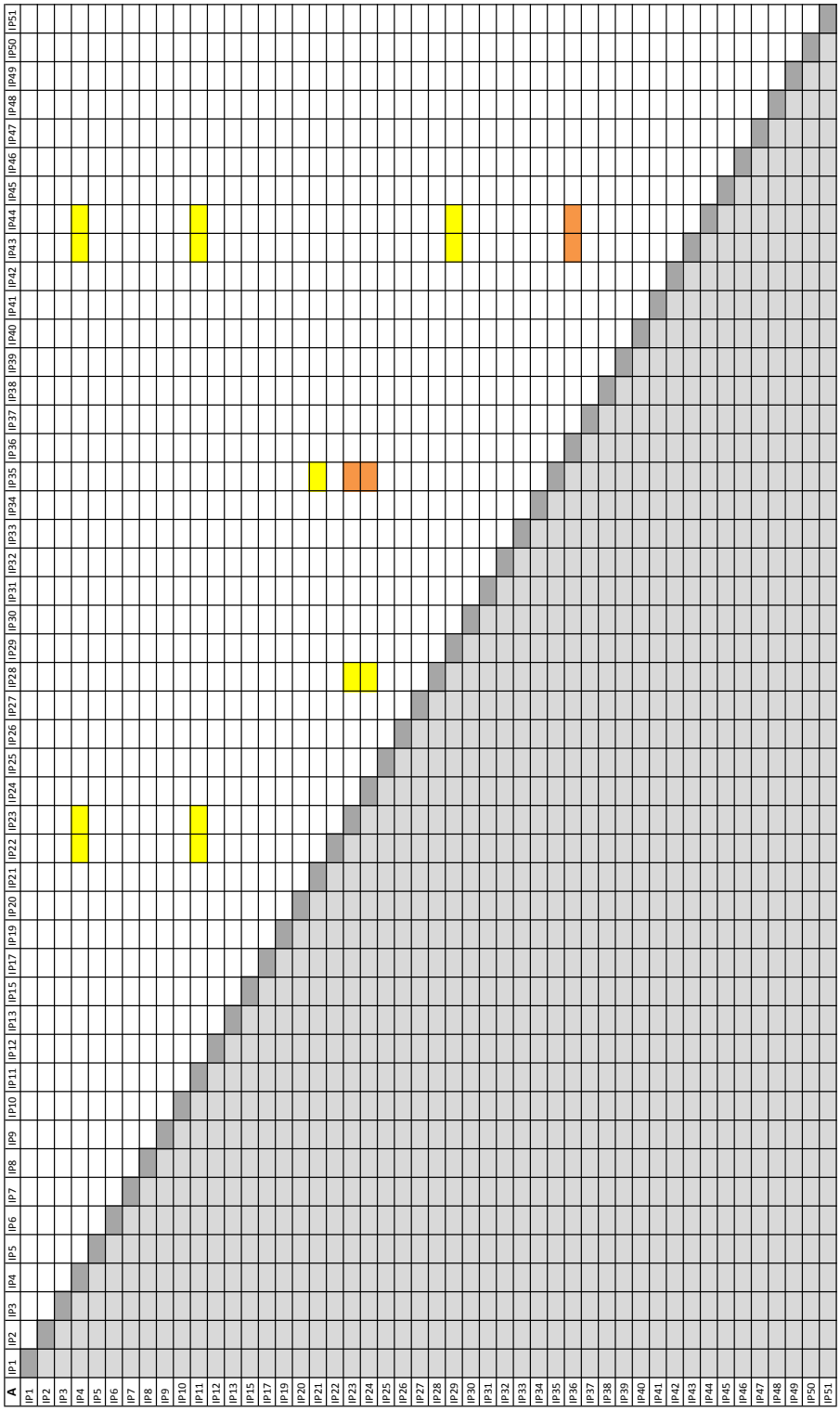
Zhao, Z. et al., 2013. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics*, **14**, 1.

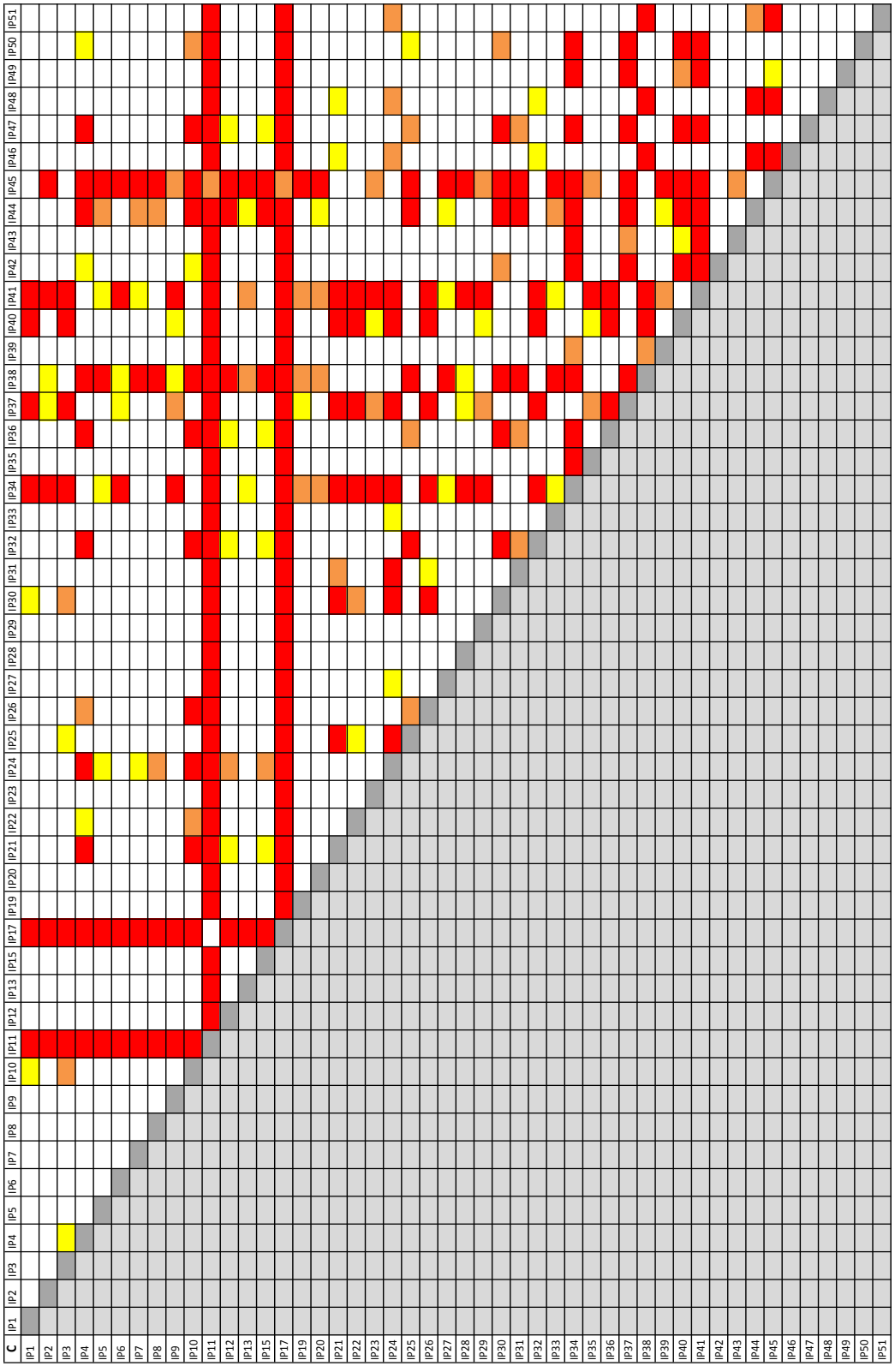
Appendix

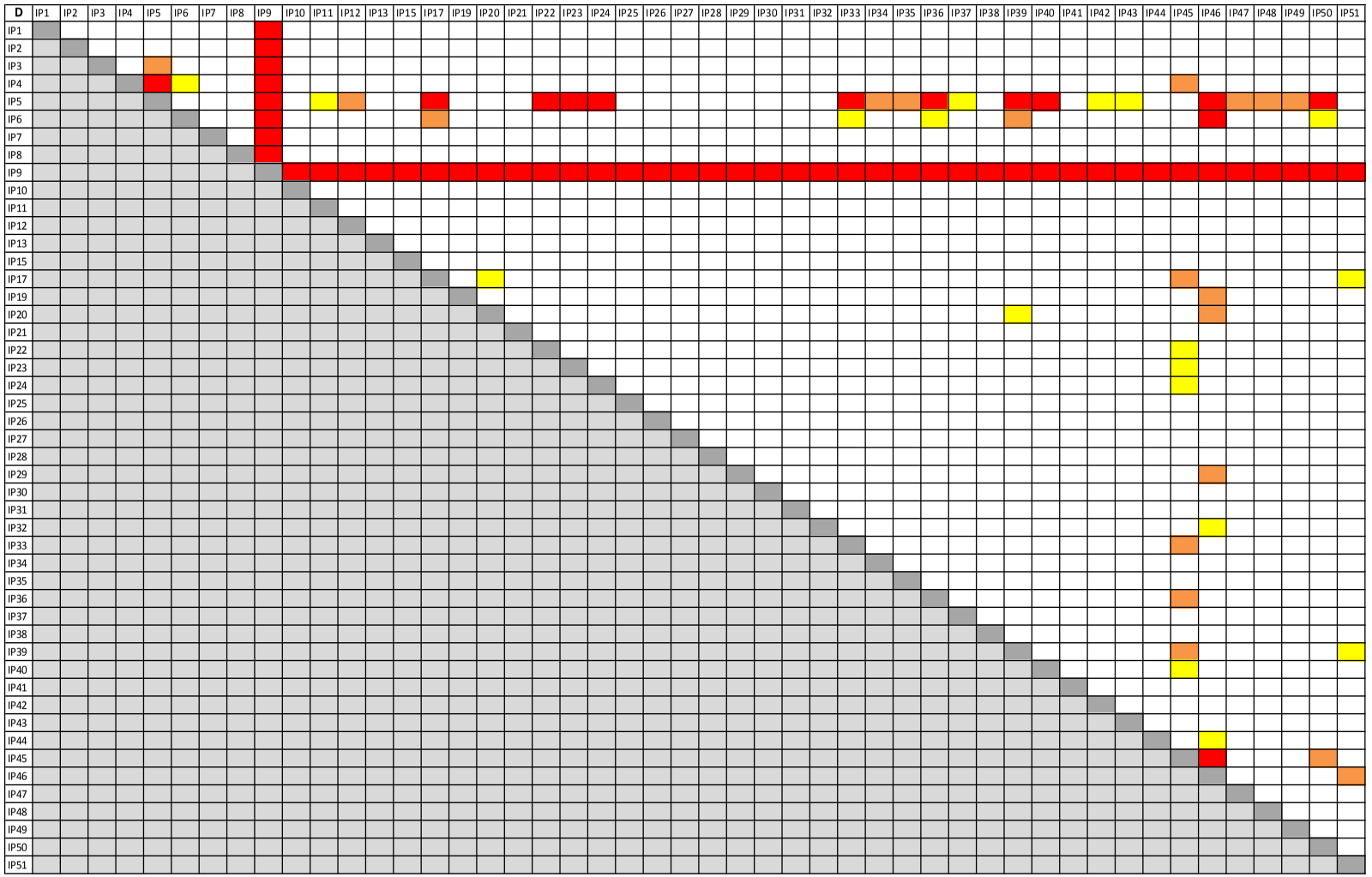
Table A.1 – Table of thermocycling conditions for the amplification of housekeeping genes for *Itersonilia* spp. isolates.

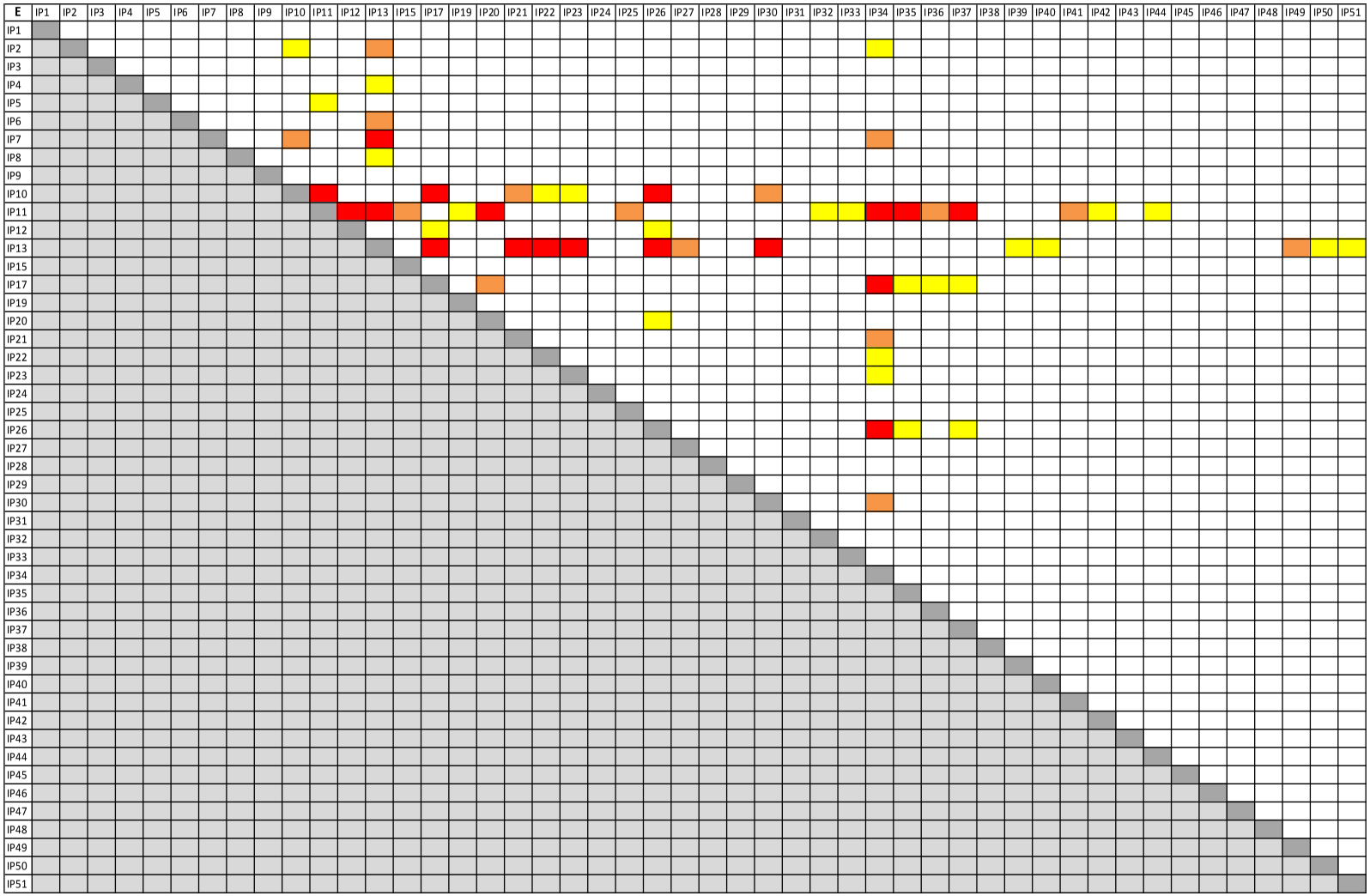
Genetic Locus		Thermocycling Conditions
ITS	Nuclear rDNA Internal transcribed spacer regions	one cycle of 2 min at 94°C, 40 cycles of 35 s at 94°C, 55 s at 61°C and 1 min at 72°C, followed by one cycle of 10 min at 72°C
EF-1α	Translation Elongation Factor 1-α	one cycle of 2 min at 94°C, 40 cycles of 35 s at 94°C, 55 s at 61°C and 1 min at 72°C, followed by one cycle of 10 min at 72°C
Rpb2	RNA Polymerase II Second largest subunit	one cycle of 2 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 68°C and 1 min at 72°C, followed by one cycle of 10 min at 72°C
TUB2	Partial β-Tubulin	one cycle of 2 min at 94°C, 35 cycles of 45 s at 94°C, 30 s at 68°C and 1 min at 72°C, followed by one cycle of 7 min at 72°C
LSU	Partial 28s rRNA gene	one cycle of 2 min at 94 °C, 30 cycles of 30 s at 94°C, 1 min at 59°C and 1 min at 72°C, followed by one cycle of 7 min at 72°C
SSU	Partial 18s rRNA gene	one cycle of 2 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 48°C and 1 min at 72°C, followed by one cycle of 7 min at 72°C
TTF	Triosephosphate Transporter Family	one cycle of 2 min at 94°C, 15 cycles of 30 s at 94°C, 1 min at 71°C (decreasing 1°C per cycle) and 1 min at 72°C, followed by 25 cycles of 30 s at 94°C, 1 min at 70°C and 1 min at 72°C followed by one cycle of 7 min at 72°C
tMT	tRNA methyl transferase	one cycle of 2 min at 94°C, 15 cycles of 30 s at 94°C, 1 min at 71°C (decreasing 1°C per cycle) and 1 min at 72°C, followed by 25 cycles of 30 s at 94°C, 1 min at 70°C and 1 min at 72°C followed by one cycle of 7 min at 72°C
CDH	Cellubiose Dehydrogenase	one cycle of 2 min at 94°C, 15 cycles of 30 s at 94°C, 1 min at 70°C (decreasing 1°C per cycle) and 1 min at 72°C, followed by 25 cycles of 30 s at 94°C, 1 min at 69°C and 1 min at 72°C followed by one cycle of 7 min at 72°C
STR	Sugar Transporter	one cycle of 2 min at 94°C, 15 cycles of 30 s at 94°C, 1 min at 70°C (decreasing 1°C per cycle) and 1 min at 72°C, followed by 25 cycles of 30 s at 94°C, 1 min at 65°C and 1 min at 72°C followed by one cycle of 7 min at 72°C

Table A.2 – Effect of temperature on *Itersonilia* spp. growth rate. Tables of 'Tukey' analysis output for all *Itersonilia* spp. isolates (IP) on malt agar (MA) from growth rate analysis at temperatures: A) 0°C, B) 5°C, C) 10°C, D) 15°C, E) 20°C and F) 25°C. Yellow indicates p<0.05, orange indicates p<0.01 and red indicates p<0.001.




C
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51


D
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51


E
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51

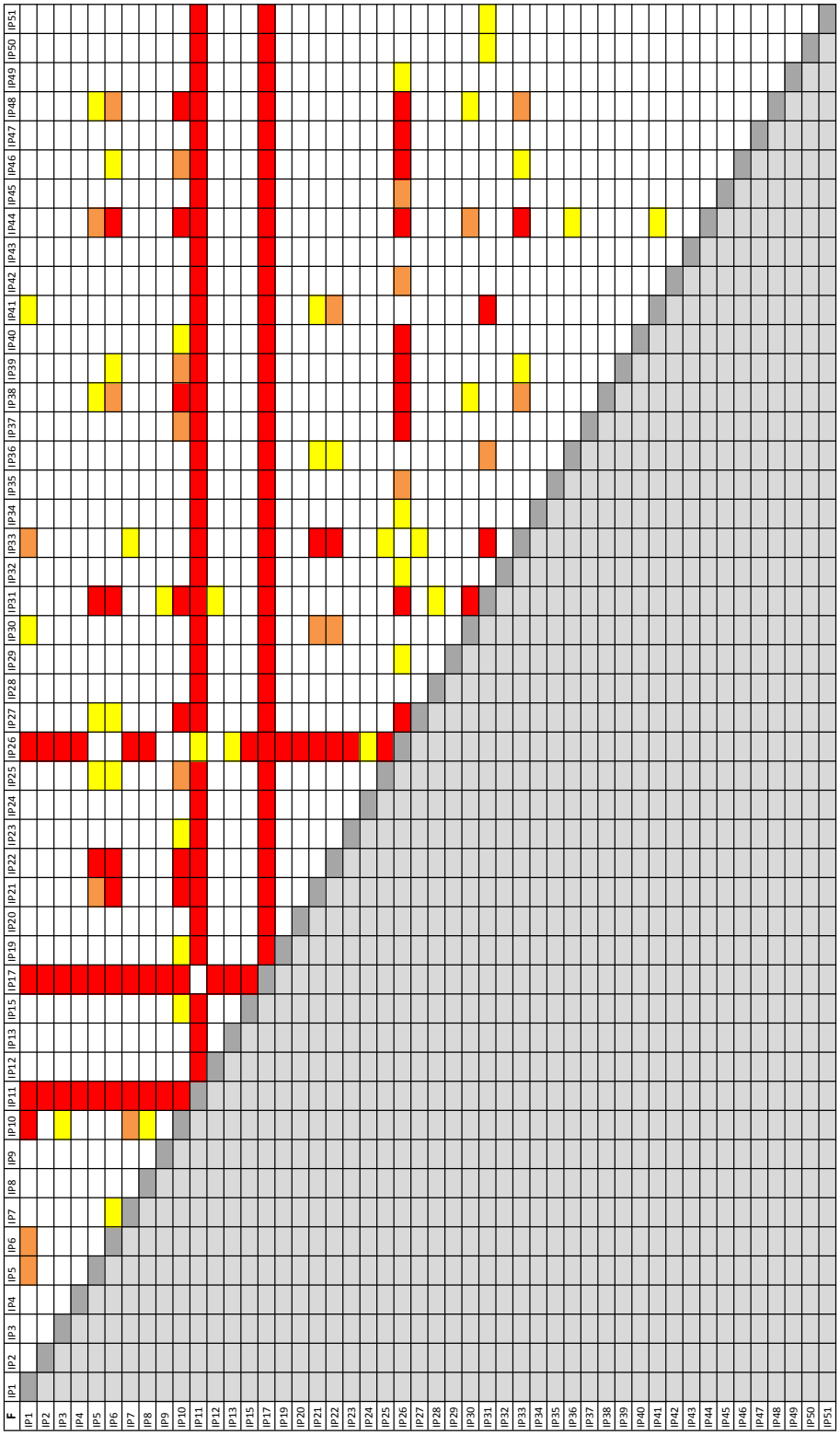
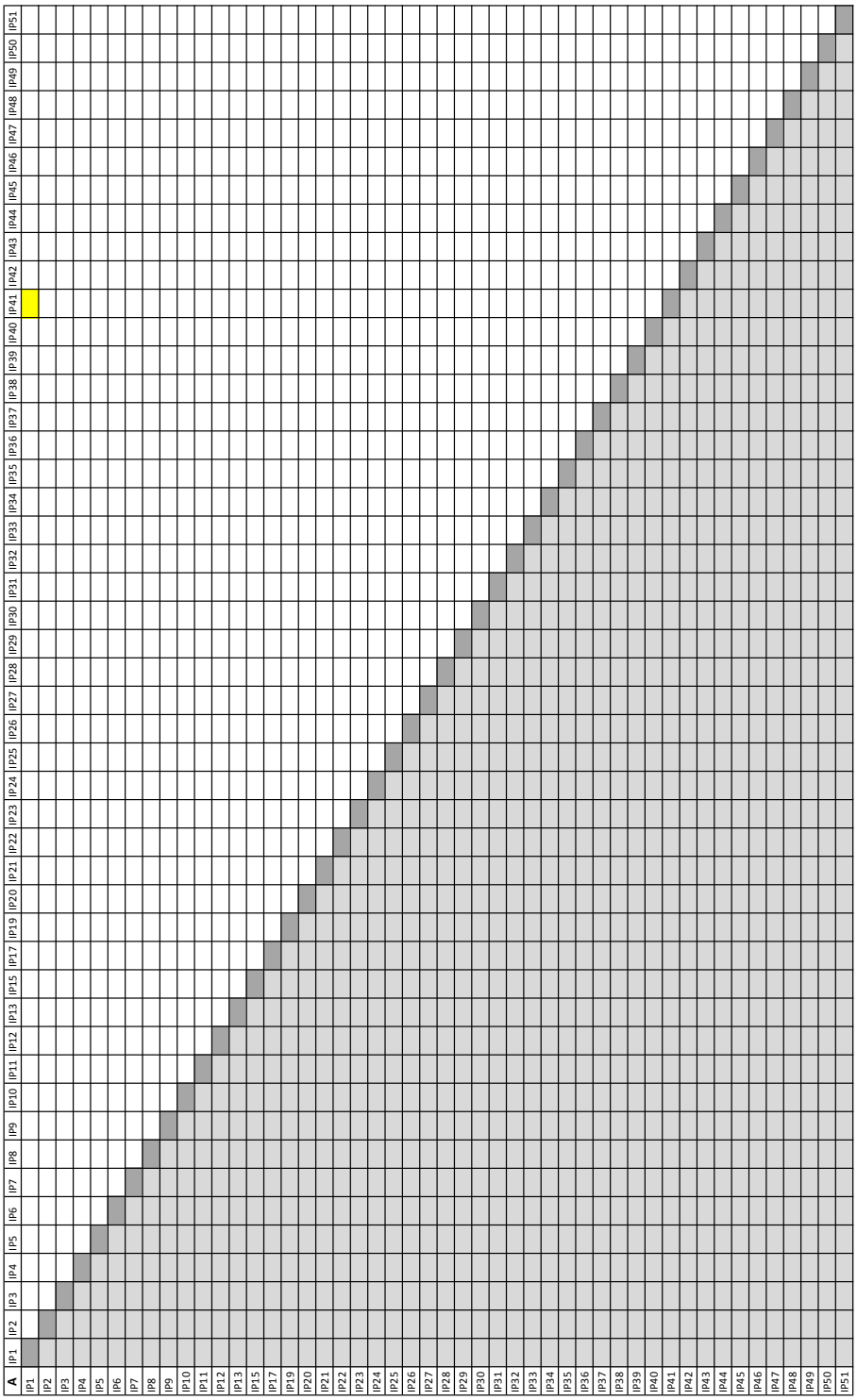
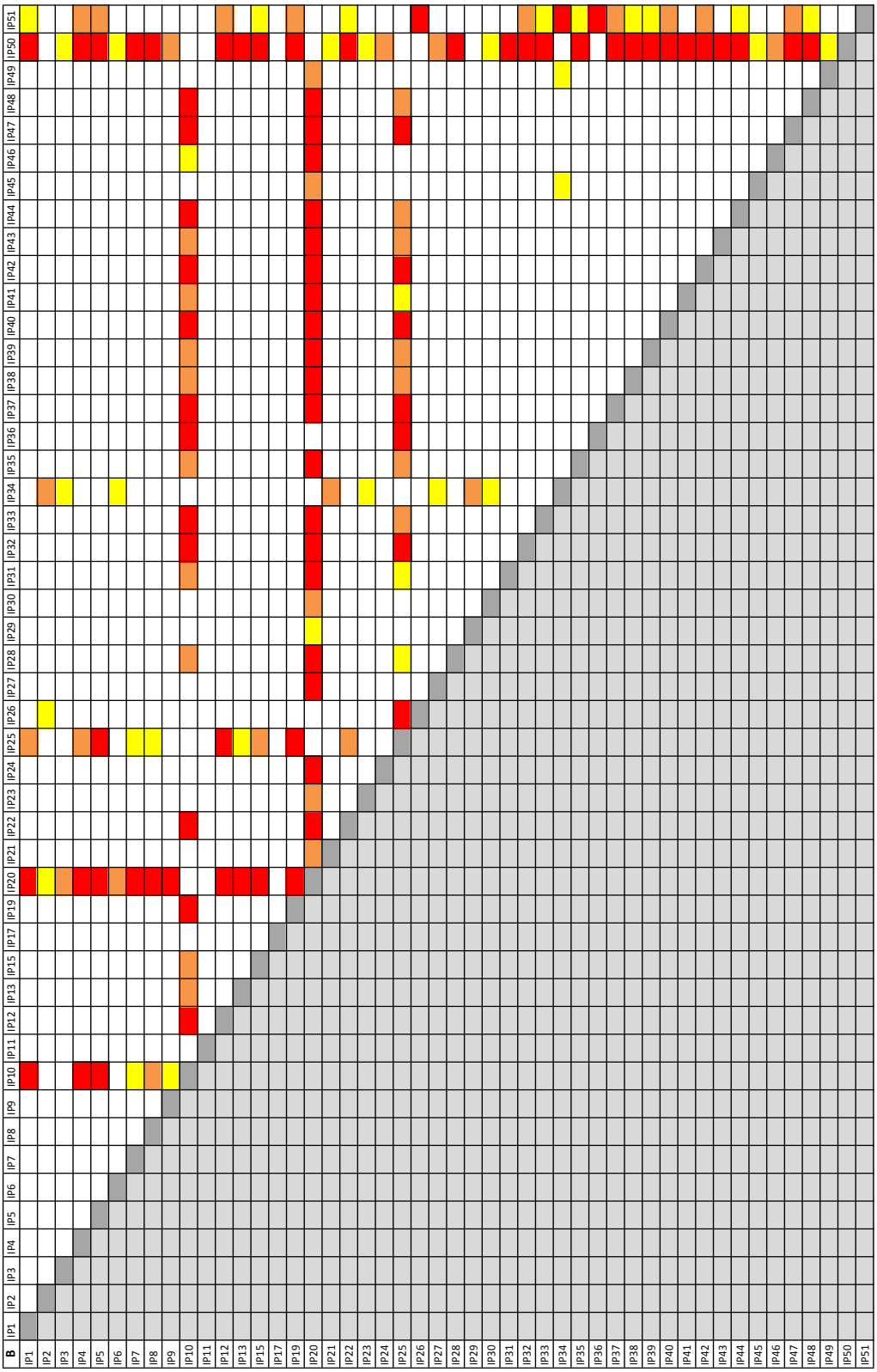
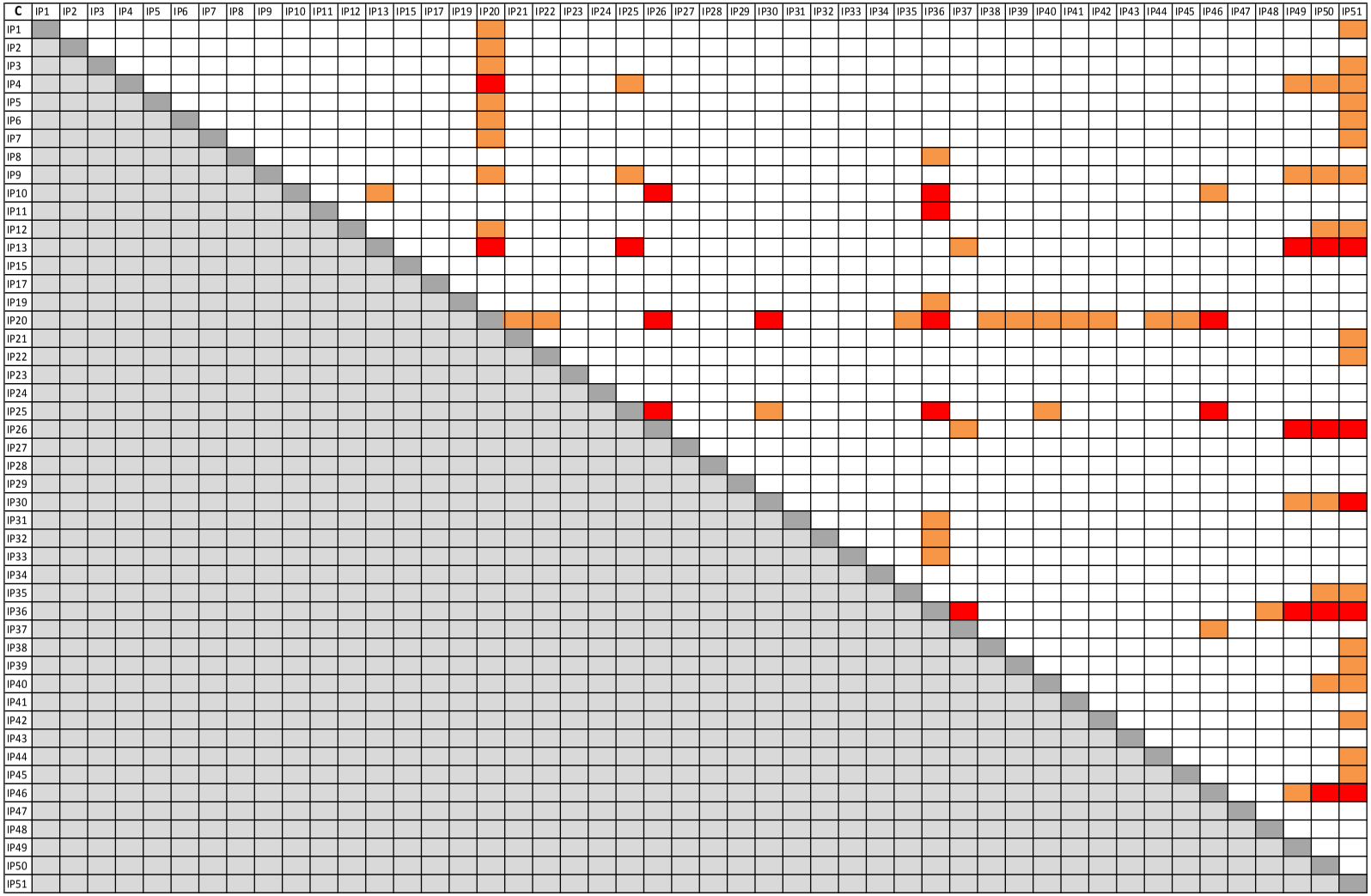
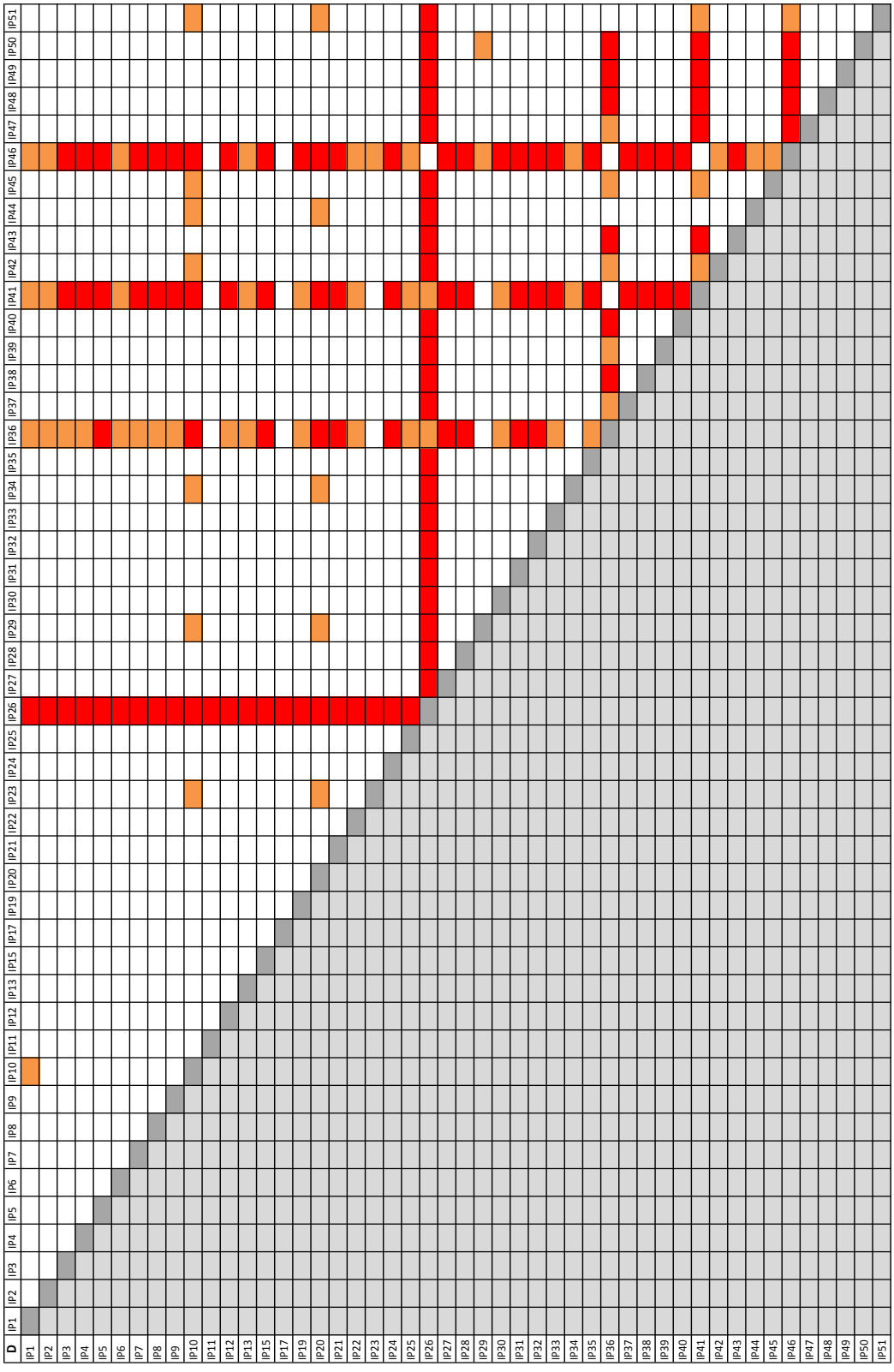

F
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51

Table A.3 – Effect of temperature on *Itersonilia* spp. spore production. Tables of 'Tukey' analysis output for all *Itersonilia* spp. isolates (IP) on malt agar (MA) from ballistospore quantification analysis at temperatures: A) 0°C, B) 10°C, C) 15°C, D) 20°C, E) 25°C. Yellow indicates p<0.05, orange indicates p<0.01 and red indicates p<0.001.




B
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51


C
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51


D
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51

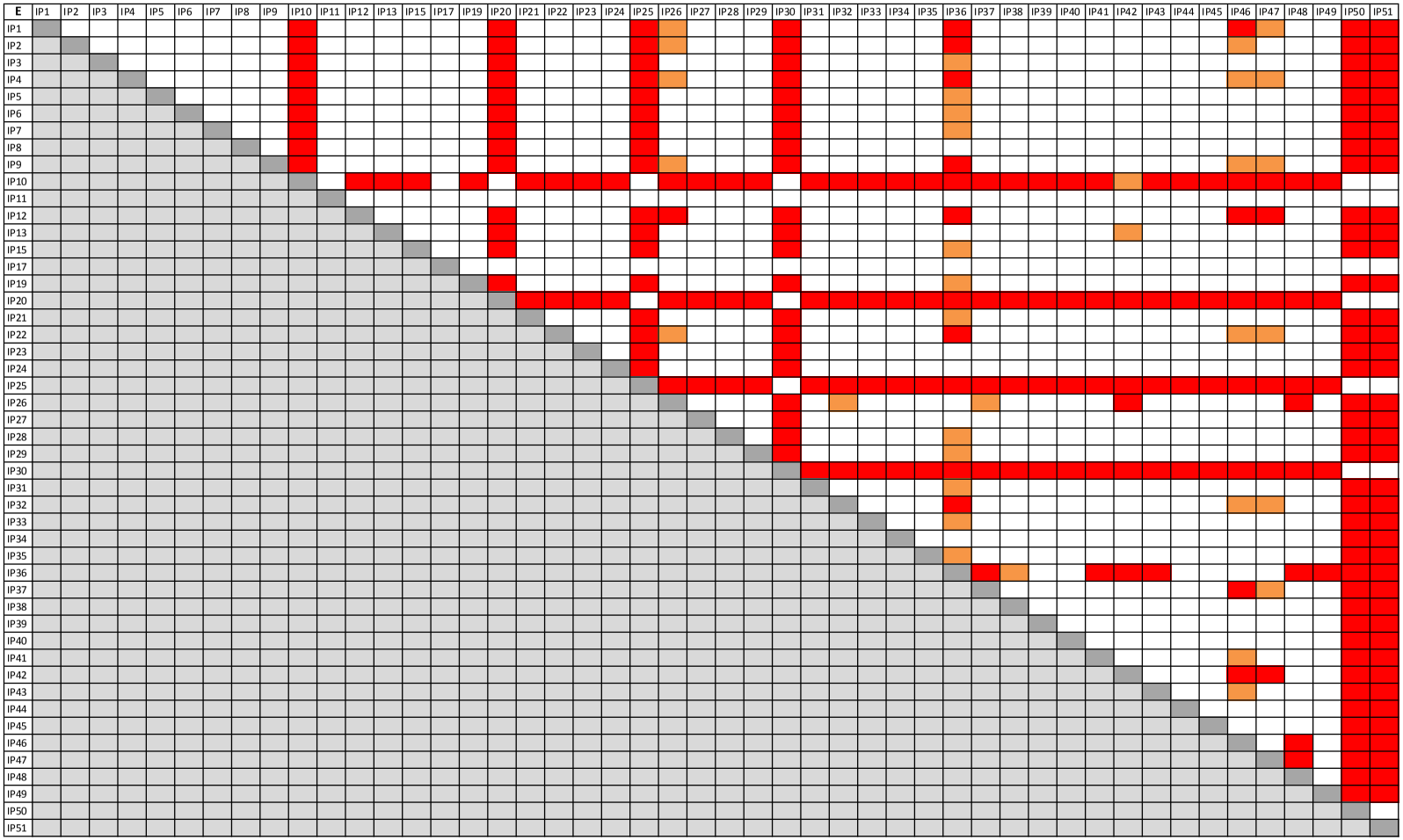

E
IP1
IP2
IP3
IP4
IP5
IP6
IP7
IP8
IP9
IP10
IP11
IP12
IP13
IP15
IP17
IP19
IP20
IP21
IP22
IP23
IP24
IP25
IP26
IP27
IP28
IP29
IP30
IP31
IP32
IP33
IP34
IP35
IP36
IP37
IP38
IP39
IP40
IP41
IP42
IP43
IP44
IP45
IP46
IP47
IP48
IP49
IP50
IP51

Table A.4 – Effect of temperature on *C. destructans* growth rate. Tables of 'Tukey' analysis output for all *C. destructans* isolates (CD) on potato dextrose agar (PDA) from growth rate analysis at temperatures: A) 5°C, B) 10°C, C) 15°C, D) 20°C and E) 25°C. Yellow indicates $p<0.05$, orange indicates $p<0.01$ and red indicates $p<0.001$.

A	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

B	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

C	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

D	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

E	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

Table A.5 – Effect of temperature on *C. destructans* growth rate. Tables of 'Tukey' analysis output for all *C. destructans* isolates (CD) on Czapek Dox agar (CDA) from growth rate analysis at temperatures: A) 5°C, B) 10°C, C) 15°C, D) 20°C and E) 25°C. Yellow indicates $p<0.05$, orange indicates $p<0.01$ and red indicates $p<0.001$.

A	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

B	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

C	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

D	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

E	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

Table A.6 – Effect of temperature on *C. destructans* spore production. Tables of 'Tukey' analysis output for all *C. destructans* isolates (CD) from spore quantification analysis at temperatures: A) 5°C, B) 10°C, C) 15°C, D) 20°C and E) 25°C. Yellow indicates $p<0.05$, orange indicates $p<0.01$ and red indicates $p<0.001$.

A	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

B	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

C	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

D	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

E	CD1	CD5	CD10	CD18	CD19	CD20	CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD30	CD31
CD1																
CD5																
CD10																
CD18																
CD19																
CD20																
CD21																
CD22																
CD23																
CD24																
CD25																
CD26																
CD27																
CD28																
CD30																
CD31																

Table A.7 – Effect of temperature on *M. acerina* growth rate. Tables of 'Tukey' analysis output for all *M. acerina* isolates (MA) from growth rate analysis at temperatures: A) 5°C, B) 10°C, C) 15°C, D) 20°C and E) 25°C. Yellow indicates $p<0.05$, orange indicates $p<0.01$ and red indicates $p<0.001$.

A	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

B	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

C	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

D	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

E	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

Table A.8 – Effect of UV light on *M. acerina* spore production. Tables of 'Tukey' analysis output for all *M. acerina* isolates (MA) from spore quantification analysis at 20°C under A) dark and B) UV light conditions. Yellow indicates p<0.05, orange indicates p<0.01 and red indicates p<0.001.

A	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

B	MA1	MA2	MA3	MA4	MA5	MA6	MA7
MA1							
MA2							
MA3							
MA4							
MA5							
MA6							
MA7							

Table A.9 – Means, 5% LSD value and corresponding significance group based on genotyping lines inoculated with *I. pastinacae* isolate IP10 from 2013/14. The degrees of freedom (d.f.) were 136.

Number	Line	Log transformed REML adjusted mean	5% LSD
1	G215	-0.9057	a
2	G66	-0.8549	ab
3	G139	-0.7749	abc
4	G216	-0.764	abcd
5	G25	-0.7619	abcd
6	G27	-0.7572	abcd
7	G211	-0.7281	abcde
8	G11	-0.7247	abcde
9	G68	-0.7093	abcde
10	G51	-0.706	abcdef
11	G86	-0.6363	abcdefg
12	G203	-0.6308	abcdefg
13	G222	-0.6284	abcdefg
14	G80	-0.627	abcdefg
15	G288	-0.6263	abcdefg
16	G28	-0.6216	abcdefg
17	G292	-0.6214	abcdefg
18	G269	-0.6211	abcdefg
19	G217	-0.6115	abcdefg
20	G207	-0.6028	abcdefg
21	G105	-0.5988	abcdefg
22	G271	-0.5954	abcdefg
23	G47	-0.5942	abcdefg
24	G287	-0.5864	abcdefg
25	G274	-0.5845	abcdefg
26	G38	-0.5742	abcdefg
27	G72	-0.5671	abcdefg
28	G183	-0.5668	abcdefg
29	G201	-0.5478	abcdefg
30	G266	-0.5477	abcdefg
31	G6	-0.5458	abcdefg
32	G286	-0.5454	abcdefg
33	G34	-0.5398	abcdefg
34	G91	-0.5379	abcdefg
35	G270	-0.5367	abcdefg
36	G206	-0.5267	abcdefg
37	G124	-0.5236	abcdefg
38	G223	-0.5129	abcdefg
39	G21	-0.5101	abcdefg
40	G280	-0.5079	abcdefg
41	G26	-0.5013	abcdefg
42	G192	-0.4981	abcdefg
43	G30	-0.4955	abcdefg
44	G19	-0.4912	abcdefg
45	G262	-0.4775	abcdefgh
46	G236	-0.4775	abcdefgh
47	G237	-0.4752	abcdefgh

Number	Line	Log transformed REML adjusted mean	5% LSD
48	G256	-0.474	abcdefgh
49	G13	-0.4735	abcdefgh
50	G54	-0.4724	abcdefgh
51	G140	-0.4708	abcdefgh
52	G190	-0.4707	abcdefgh
53	G144	-0.4637	abcdefgh
54	G258	-0.4633	abcdefgh
55	G31	-0.4596	abcdefgh
56	G89	-0.4589	abcdefgh
57	G234	-0.4529	abcdefgh
58	G153	-0.4509	abcdefgh
59	G199	-0.4503	abcdefgh
60	G15	-0.4478	abcdefgh
61	G268	-0.4466	abcdefgh
62	G79	-0.4436	abcdefgh
63	G273	-0.4434	abcdefgh
64	G77	-0.4427	abcdefgh
65	G251	-0.4389	abcdefgh
66	G119	-0.4367	abcdefgh
67	G103	-0.4366	abcdefgh
68	G108	-0.4324	abcdefgh
69	G35	-0.4311	abcdefgh
70	G1	-0.4275	abcdefgh
71	G62	-0.4275	abcdefgh
72	G69	-0.4275	abcdefgh
73	G221	-0.423	abcdefgh
74	G240	-0.4221	abcdefgh
75	G57	-0.4202	abcdefgh
76	G63	-0.4152	abcdefgh
77	G159	-0.408	abcdefgh
78	G249	-0.407	abcdefgh
79	G20	-0.4049	abcdefgh
80	G220	-0.4025	abcdefgh
81	G282	-0.4025	abcdefgh
82	G277	-0.3917	abcdefgh
83	G291	-0.3912	abcdefgh
84	G197	-0.3907	abcdefgh
85	G82	-0.3817	abcdefgh
86	G143	-0.3796	abcdefgh
87	G5	-0.3786	abcdefgh
88	G111	-0.3765	abcdefgh
89	G245	-0.3706	abcdefgh
90	G278	-0.3699	abcdefgh
91	G75	-0.3691	abcdefgh
92	G36	-0.3631	abcdefgh
93	G263	-0.3626	abcdefgh
94	G7	-0.358	abcdefgh
95	G233	-0.3575	abcdefgh
96	G259	-0.354	abcdefgh
97	G48	-0.3531	abcdefgh
98	G151	-0.352	abcdefgh
99	G142	-0.338	abcdefgh

Number	Line	Log transformed REML adjusted mean	5% LSD
100	G32	-0.3344	abcdefgh
101	G46	-0.3341	abcdefgh
102	G14	-0.3267	abcdefgh
103	G92	-0.3261	abcdefgh
104	G60	-0.3247	abcdefgh
105	G152	-0.3098	abcdefgh
106	G87	-0.2977	abcdefgh
107	G49	-0.2967	abcdefgh
108	G253	-0.2954	abcdefgh
109	G196	-0.2936	abcdefgh
110	G289	-0.2894	abcdefgh
111	G138	-0.2852	abcdefgh
112	G17	-0.2791	abcdefgh
113	G227	-0.2787	abcdefgh
114	G225	-0.2785	abcdefgh
115	G64	-0.276	bcdefgh
116	G219	-0.2726	bcdefgh
117	G230	-0.2685	bcdefgh
118	G184	-0.2647	bcdefgh
119	G3	-0.2582	bcdefgh
120	G39	-0.251	bcdefgh
121	G239	-0.251	bcdefgh
122	G281	-0.2472	bcdefgh
123	G243	-0.2321	bcdefgh
124	G210	-0.2314	bcdefgh
125	G59	-0.2234	cdefgh
126	G279	-0.2207	cdefgh
127	G218	-0.2159	cdefgh
128	G76	-0.2157	cdefgh
129	G248	-0.1956	cdefgh
130	G250	-0.1722	cdefgh
131	G257	-0.1609	cdefgh
132	G65	-0.1511	cdefgh
133	G267	-0.1478	cdefgh
134	G261	-0.1465	defgh
135	G247	-0.1233	efgh
136	G241	-0.0788	fgh
137	G212	-0.0628	gh
138	G58	0.1452	h
d.f.	136		
5% LSD	0.31035		

Table A.10 – Means, 5% LSD value and corresponding significance group based on genotyping lines inoculated with *I. pastinacae* isolate IP10 from 2014/15. The degrees of freedom (d.f.) were 137.

Number	Line	Log transformed REML adjusted mean	5% LSD
1	G211	-0.566	a
2	G80	-0.5306	ab
3	G271	-0.5306	ab
4	G245	-0.5212	ab
5	G288	-0.5067	abc
6	G159	-0.4763	abc
7	G190	-0.4739	abc
8	G227	-0.472	abc
9	G291	-0.4704	abc
10	G219	-0.4557	abc
11	G274	-0.4469	abc
12	G223	-0.4441	abc
13	G270	-0.4428	abc
14	G210	-0.4397	abc
15	G233	-0.4345	abc
16	G230	-0.4294	abc
17	G266	-0.4233	abc
18	G289	-0.4208	abc
19	G108	-0.4147	abc
20	G25	-0.409	abc
21	G259	-0.3975	abc
22	G21	-0.3951	abc
23	G262	-0.3933	abc
24	G267	-0.393	abc
25	G199	-0.3922	abc
26	G38	-0.3879	abc
27	G221	-0.3867	abc
28	G248	-0.3716	abc
29	G249	-0.3622	abc
30	G103	-0.3532	abc
31	G152	-0.339	abc
32	G279	-0.3369	abc
33	G28	-0.3335	abc
34	G225	-0.326	abc
35	G140	-0.3236	abc
36	G124	-0.3203	abc
37	G68	-0.3191	abc
38	G286	-0.3183	abc
39	G236	-0.3161	abc
40	G119	-0.3122	abc
41	G222	-0.3111	abc
42	G142	-0.308	abc
43	G261	-0.3059	abc
44	G48	-0.3051	abc
45	G89	-0.3022	abc
46	G184	-0.3003	abc
47	G91	-0.2961	abc

Number	Line	Log transformed REML adjusted mean	5% LSD
48	G216	-0.2942	abc
49	G19	-0.2939	abc
50	G257	-0.2927	abc
51	G256	-0.2837	abc
52	G59	-0.2836	abc
53	G217	-0.2748	abc
54	G269	-0.2741	abc
55	G111	-0.2691	abc
56	G218	-0.2649	abcd
57	G64	-0.2604	abcd
58	G239	-0.2585	abcd
59	G212	-0.2514	abcde
60	G282	-0.2492	abcde
61	G36	-0.2469	abcde
62	G47	-0.2414	abcde
63	G26	-0.2368	abcde
64	G278	-0.2322	abcde
65	G139	-0.2312	abcde
66	G250	-0.2234	abcde
67	G14	-0.2224	abcde
68	G263	-0.2187	abcde
69	G143	-0.2178	abcde
70	G72	-0.2175	abcde
71	G151	-0.2174	abcde
72	G241	-0.2141	abcde
73	G273	-0.2097	abcde
74	G144	-0.2058	abcde
75	G201	-0.2053	abcde
76	G196	-0.2049	abcde
77	G13	-0.1993	abcde
78	G215	-0.1991	abcde
79	G197	-0.1959	abcde
80	G39	-0.1591	abcde
81	G86	-0.1392	abcde
82	G35	-0.133	abcde
83	G57	-0.1223	abcde
84	G277	-0.1216	abcde
85	G54	-0.1191	abcde
86	G63	-0.107	abcde
87	G207	-0.1039	abcde
88	G6	-0.1035	abcde
89	G34	-0.0949	abcde
90	G105	-0.0942	abcde
91	G27	-0.084	abcde
92	G206	-0.0737	abcde
93	G258	-0.073	abcde
94	G20	-0.0713	abcde
95	G243	-0.0684	abcde
96	G76	-0.0671	abcde
97	G51	-0.0652	abcde
98	G77	-0.0593	abcde
99	G30	-0.0577	abcde

Number	Line	Log transformed REML adjusted mean	5% LSD
100	G292	-0.0553	abcde
101	G69	-0.0542	abcde
102	G203	-0.054	abcde
103	G66	-0.0539	abcde
104	G220	-0.0466	abcde
105	G75	-0.0393	abcde
106	G237	-0.0373	abcde
107	G31	-0.0353	abcde
108	G280	-0.0331	abcde
109	G287	-0.03	abcde
110	G240	-0.0239	abcde
111	G92	-0.0182	abcde
112	G1	-0.0028	abcde
113	G251	-0.0026	abcde
114	G138	0.0006	abcde
115	G32	0.0041	abcde
116	G11	0.0263	abcde
117	G87	0.03	abcde
118	G60	0.0339	abcde
119	G65	0.0355	abcde
120	G253	0.036	abcde
121	G62	0.0449	abcde
122	G153	0.051	abcde
123	G49	0.0532	abcde
124	G17	0.0538	abcde
125	G268	0.0604	abcde
126	G192	0.0878	abcde
127	G82	0.0892	abcde
128	G7	0.1002	abcde
129	G234	0.1039	abcde
130	G46	0.1184	abcde
131	G5	0.1189	abcde
132	G281	0.1364	abcde
133	G15	0.148	abcde
134	G3	0.1616	abcde
135	G183	0.2081	bcde
136	G58	0.2606	cde
137	G247	0.5037	de
138	G79	0.5228	e
d.f.	137		
5% LSD	0.3829		

Table A.11 – Means, 5% LSD value and corresponding significance group based on genotyping lines inoculated *M. acerina* isolate MA2 from 2013/14. The degrees of freedom (d.f.) were 137.

Number	Line	Log transformed REML adjusted mean	5% LSD
1	G196	-0.6435	a
2	G77	-0.616	ab
3	G217	-0.6079	abc
4	G66	-0.5971	abcd
5	G72	-0.5971	abcd
6	G89	-0.5626	abcde
7	G223	-0.5604	abcde
8	G207	-0.5546	abcde
9	G286	-0.5255	abcdef
10	G80	-0.5245	abcdef
11	G17	-0.5229	abcdef
12	G263	-0.5127	abcdefg
13	G28	-0.5121	abcdefg
14	G203	-0.506	abcdefg
15	G221	-0.4925	abcdefg
16	G140	-0.4852	abcdefg
17	G287	-0.4759	abcdefgh
18	G266	-0.4751	abcdefgh
19	G138	-0.4725	abcdefgh
20	G240	-0.4715	abcdefgh
21	G68	-0.4706	abcdefghi
22	G75	-0.4706	abcdefghi
23	G82	-0.4642	abcdefghi
24	G20	-0.4596	abcdefghi
25	G142	-0.4595	abcdefghi
26	G32	-0.4573	abcdefghi
27	G108	-0.4475	abcdefghi
28	G38	-0.4471	abcdefghi
29	G259	-0.4419	abcdefghi
30	G268	-0.4319	abcdefghi
31	G87	-0.4279	abcdefghi
32	G197	-0.4195	abcdefghi
33	G237	-0.4174	abcdefghi
34	G201	-0.4059	abcdefghi
35	G36	-0.3978	abcdefghi
36	G292	-0.3956	abcdefghi
37	G251	-0.3941	abcdefghi
38	G289	-0.394	abcdefghi
39	G216	-0.3937	abcdefghi
40	G47	-0.3829	abcdefghi
41	G34	-0.3828	abcdefghi
42	G212	-0.3786	abcdefghi
43	G91	-0.3755	abcdefghi
44	G227	-0.3751	abcdefghi
45	G261	-0.3697	abcdefghi
46	G49	-0.3688	abcdefghi
47	G105	-0.3587	abcdefghi

Number	Line	Log transformed REML adjusted mean	5% LSD
48	G249	-0.349	abcdefghi
49	G6	-0.3471	abcdefghi
50	G262	-0.3455	abcdefghi
51	G124	-0.3444	abcdefghi
52	G269	-0.3362	abcdefghi
53	G219	-0.3297	abcdefghi
54	G119	-0.3258	abcdefghi
55	G288	-0.3196	abcdefghi
56	G143	-0.2871	abcdefghi
57	G159	-0.2833	abcdefghi
58	G236	-0.2813	abcdefghi
59	G271	-0.2798	abcdefghi
60	G273	-0.277	abcdefghi
61	G46	-0.2717	abcdefghi
62	G144	-0.269	abcdefghi
63	G86	-0.2657	abcdefghi
64	G31	-0.2653	abcdefghi
65	G258	-0.2502	abcdefghij
66	G278	-0.2445	abcdefghij
67	G27	-0.2404	abcdefghij
68	G25	-0.24	abcdefghij
69	G239	-0.2387	abcdefghij
70	G139	-0.2376	abcdefghij
71	G215	-0.2375	abcdefghij
72	G190	-0.2373	abcdefghij
73	G250	-0.2338	abcdefghij
74	G62	-0.2329	abcdefghij
75	G7	-0.2329	abcdefghij
76	G220	-0.2302	abcdefghij
77	G274	-0.228	abcdefghij
78	G3	-0.2087	abcdefghij
79	G253	-0.2083	abcdefghij
80	G13	-0.1985	abcdefghij
81	G245	-0.1973	abcdefghij
82	G241	-0.1922	abcdefghij
83	G111	-0.1889	abcdefghij
84	G233	-0.181	abcdefghij
85	G199	-0.1692	abcdefghij
86	G248	-0.1601	abcdefghij
87	G234	-0.1597	abcdefghij
88	G210	-0.1597	abcdefghij
89	G247	-0.1569	abcdefghij
90	G15	-0.1551	abcdefghij
91	G281	-0.1518	abcdefghij
92	G257	-0.1473	abcdefghij
93	G291	-0.1409	abcdefghij
94	G64	-0.1361	abcdefghij
95	G279	-0.1336	abcdefghij
96	G267	-0.1179	abcdefghij
97	G183	-0.1004	abcdefghij
98	G5	-0.0996	abcdefghij
99	G30	-0.0964	abcdefghij

Number	Line	Log transformed REML adjusted mean	5% LSD
100	G206	-0.0953	abcdefghij
101	G270	-0.0854	abcdefghij
102	G92	-0.0788	abcdefghij
103	G76	-0.0766	abcdefghij
104	G14	-0.0731	abcdefghij
105	G35	-0.0709	abcdefghij
106	G39	-0.0607	abcdefghij
107	G21	-0.0486	abcdefghij
108	G256	-0.0454	abcdefghij
109	G1	-0.0428	abcdefghij
110	G19	-0.036	abcdefghij
111	G277	-0.0322	abcdefghij
112	G54	-0.0313	abcdefghij
113	G153	-0.0304	abcdefghij
114	G152	-0.0287	abcdefghij
115	G65	-0.0246	abcdefghij
116	G280	-0.0114	abcdefghij
117	G222	-0.0084	abcdefghij
118	G51	0.003	abcdefghij
119	G184	0.0051	abcdefghij
120	G59	0.0149	abcdefghij
121	G63	0.0158	abcdefghij
122	G243	0.0476	bcdefghij
123	G282	0.0496	bcdefghij
124	G103	0.0527	bcdefghij
125	G48	0.0641	bcdefghij
126	G211	0.0673	cdefghij
127	G230	0.083	defghij
128	G57	0.0873	efghij
129	G26	0.0987	efghij
130	G11	0.1317	fghij
131	G218	0.1393	fghij
132	G225	0.1475	fghij
133	G60	0.1532	fghij
134	G69	0.1687	ghij
135	G79	0.1995	hij
136	G192	0.2007	hij
137	G151	0.2114	ij
138	G58	0.4263	j
d.f.	137		
5% LSD	0.2923		

Table A.12 – Means, 5% LSD value and corresponding significance group based on genotyping lines inoculated with *M. acerina* isolate MA2 from 2014/15. The degrees of freedom (d.f.) were 137.

Number	Line	Log transformed REML adjusted mean	5% LSD
1	G257	-0.3245	a
2	G289	-0.3197	ab
3	G216	-0.2635	abc
4	G221	-0.2529	abc
5	G190	-0.2102	abc
6	G201	-0.1981	abc
7	G199	-0.1955	abc
8	G233	-0.173	abc
9	G249	-0.1582	abc
10	G245	-0.1565	abc
11	G271	-0.1542	abc
12	G280	-0.1454	abcd
13	G219	-0.1449	abcd
14	G192	-0.1385	abcd
15	G60	-0.1304	abcd
16	G230	-0.1284	abcd
17	G196	-0.1226	abcd
18	G142	-0.1199	abcd
19	G269	-0.1196	abcd
20	G210	-0.1179	abcd
21	G286	-0.1119	abcd
22	G46	-0.1066	abcd
23	G279	-0.1058	abcd
24	G91	-0.0934	abcd
25	G32	-0.0926	abcd
26	G211	-0.0846	abcd
27	G57	-0.0807	abcde
28	G144	-0.0782	abcde
29	G87	-0.0671	abcde
30	G31	-0.0651	abcde
31	G159	-0.0637	abcde
32	G262	-0.0619	abcde
33	G273	-0.0591	abcde
34	G263	-0.054	abcde
35	G27	-0.0521	abcde
36	G66	-0.0472	abcde
37	G197	-0.0412	abcde
38	G278	-0.0332	abcde
39	G267	-0.0325	abcde
40	G58	-0.0302	abcde
41	G119	-0.0282	abcde
42	G17	-0.0145	abcde
43	G268	-0.0137	abcde
44	G21	-0.013	abcde
45	G86	-0.0123	abcde
46	G291	-0.0088	abcde
47	G207	-0.0009	abcde

Number	Line	Log transformed REML adjusted mean	5% LSD
48	G25	0.0001	abcde
49	G218	0.0022	abcde
50	G39	0.0025	abcde
51	G5	0.0093	abcde
52	G26	0.0108	abcde
53	G64	0.0128	abcde
54	G3	0.0141	abcde
55	G151	0.0155	abcde
56	G223	0.0218	abcde
57	G38	0.0235	abcde
58	G217	0.0242	abcde
59	G36	0.0246	abcde
60	G92	0.0264	abcde
61	G47	0.03	abcde
62	G236	0.0302	abcde
63	G274	0.0308	abcde
64	G105	0.0323	abcde
65	G243	0.0329	abcde
66	G28	0.0343	abcde
67	G76	0.0371	abcde
68	G124	0.0373	abcde
69	G225	0.0414	abcde
70	G227	0.0431	abcde
71	G140	0.0445	abcde
72	G258	0.048	abcde
73	G234	0.0538	abcde
74	G82	0.0573	abcde
75	G68	0.0599	abcde
76	G261	0.0609	abcde
77	G251	0.0623	abcde
78	G240	0.0628	abcde
79	G49	0.0642	abcde
80	G69	0.0657	abcde
81	G292	0.0671	abcde
82	G103	0.0696	abcde
83	G72	0.0703	abcde
84	G287	0.0723	abcde
85	G54	0.0749	abcde
86	G256	0.0765	abcde
87	G270	0.0772	abcde
88	G206	0.0795	abcde
89	G80	0.0855	abcde
90	G89	0.0882	abcde
91	G215	0.0897	abcde
92	G239	0.096	abcde
93	G1	0.1095	abcde
94	G288	0.1221	abcde
95	G143	0.1226	abcde
96	G108	0.123	abcde
97	G111	0.1311	abcde
98	G34	0.1321	abcde
99	G13	0.1542	abcde

Number	Line	Log transformed REML adjusted mean	5% LSD
100	G77	0.1598	abcde
101	G248	0.1697	abcde
102	G139	0.1704	abcde
103	G30	0.1762	abcde
104	G259	0.179	abcde
105	G75	0.1842	abcde
106	G19	0.1975	abcde
107	G20	0.2065	abcde
108	G153	0.2073	abcde
109	G152	0.2108	abcde
110	G35	0.2195	abcde
111	G183	0.2238	abcde
112	G63	0.2317	abcde
113	G250	0.2344	abcde
114	G266	0.2352	abcde
115	G281	0.2356	abcde
116	G237	0.2425	abcde
117	G51	0.2459	abcde
118	G11	0.2523	abcde
119	G138	0.2615	abcde
120	G48	0.268	abcde
121	G62	0.2711	abcde
122	G253	0.2747	abcde
123	G7	0.2819	abcde
124	G59	0.2833	abcde
125	G184	0.285	abcde
126	G6	0.2904	abcde
127	G277	0.2916	abcde
128	G220	0.2926	abcde
129	G15	0.2936	abcde
130	G212	0.3014	abcde
131	G222	0.3065	abcde
132	G282	0.3124	abcde
133	G203	0.3242	abcde
134	G241	0.3436	bcde
135	G79	0.3854	cde
136	G247	0.3999	cde
137	G14	0.5138	de
138	G65	0.5825	e
d.f.	137		
5% LSD	0.28561		

Table A.13 – Means, 5% LSD value and corresponding significance group based on genotyping lines inoculated with *M. acerina* isolate MA2 from 2015/16. The degrees of freedom (d.f.) were 126.

Number	Line	Log transformed REML adjusted mean	5% LSD
1	G267	-0.537	a
2	G268	-0.513	ab
3	G28	-0.4664	abc
4	G62	-0.4491	abcd
5	G46	-0.4264	abcd
6	G80	-0.4082	abcde
7	G35	-0.3843	abcdef
8	G77	-0.3674	abcdef
9	G207	-0.3501	abcdef
10	G19	-0.321	abcdefg
11	G219	-0.3083	abcdefgh
12	G201	-0.2999	abcdefgh
13	G39	-0.2848	abcdefghi
14	G217	-0.2798	abcdefghi
15	G221	-0.2728	abcdefghi
16	G60	-0.26	abcdefghi
17	G3	-0.2582	abcdefghij
18	G266	-0.2275	abcdefghijk
19	G27	-0.2187	abcdefghijk
20	G273	-0.1817	abcdefghijk
21	G196	-0.1811	abcdefghijk
22	G279	-0.1808	abcdefghijk
23	G48	-0.1796	abcdefghijk
24	G280	-0.1789	abcdefghijk
25	G5	-0.1764	abcdefghijk
26	G151	-0.1756	abcdefghijk
27	G220	-0.1694	abcdefghijk
28	G20	-0.1581	abcdefghijk
29	G79	-0.1562	abcdefghijk
30	G15	-0.1509	abcdefghijk
31	G278	-0.1492	abcdefghijk
32	G245	-0.1373	abcdefghijk
33	G269	-0.1339	abcdefghijk
34	G199	-0.126	abcdefghijk
35	G11	-0.1183	abcdefghijk
36	G227	-0.1094	abcdefghijk
37	G223	-0.1087	abcdefghijk
38	G257	-0.106	abcdefghijk
39	G139	-0.0835	abcdefghijk
40	G210	-0.0801	abcdefghijk
41	G263	-0.0725	abcdefghijk
42	G197	-0.0672	abcdefghijk
43	G54	-0.06	abcdefghijk
44	G144	-0.0513	abcdefghijk
45	G13	-0.0362	abcdefghijk
46	G152	-0.0237	abcdefghijk
47	G270	-0.0106	abcdefghijk

Number	Line	Log transformed REML adjusted mean	5% LSD
48	G183	-0.0095	abcdefghijk
49	G203	-0.0074	abcdefghijk
50	G65	-0.0071	abcdefghijk
51	G253	-0.0067	abcdefghijk
52	G271	-0.0022	abcdefghijk
53	G222	0.0137	abcdefghijk
54	G31	0.0145	abcdefghijk
55	G190	0.0171	abcdefghijk
56	G287	0.0272	abcdefghijk
57	G59	0.029	abcdefghijk
58	G14	0.0455	abcdefghijk
59	G17	0.0473	abcdefghijk
60	G206	0.0473	abcdefghijk
61	G212	0.0473	abcdefghijk
62	G261	0.0473	abcdefghijk
63	G262	0.0473	abcdefghijk
64	G30	0.0473	abcdefghijk
65	G47	0.0473	abcdefghijk
66	G68	0.0473	abcdefghijk
67	G91	0.0473	abcdefghijk
68	G89	0.0577	abcdefghijk
69	G103	0.0705	abcdefghijk
70	G216	0.0727	abcdefghijk
71	G286	0.0761	abcdefghijk
72	G86	0.0917	abcdefghijk
73	G51	0.0932	abcdefghijk
74	G66	0.1064	abcdefghijk
75	G233	0.1126	abcdefghijk
76	G140	0.1137	abcdefghijk
77	G159	0.1157	abcdefghijk
78	G76	0.1204	abcdefghijk
79	G289	0.1208	abcdefghijk
80	G258	0.1273	abcdefghijk
81	G119	0.1276	abcdefghijk
82	G111	0.1389	abcdefghijk
83	G58	0.1444	abcdefghijk
84	G256	0.1479	abcdefghijk
85	G138	0.1489	abcdefghijk
86	G274	0.1516	abcdefghijk
87	G32	0.1532	abcdefghijk
88	G236	0.1574	abcdefghijk
89	G237	0.1652	abcdefghijk
90	G259	0.1672	abcdefghijk
91	G218	0.1817	abcdefghijk
92	G230	0.1844	abcdefghijk
93	G153	0.1891	abcdefghijk
94	G108	0.1931	abcdefghijk
95	G215	0.1938	abcdefghijk
96	G49	0.1965	abcdefghijk
97	G1	0.199	abcdefghijk
98	G250	0.2055	abcdefghijk
99	G184	0.2103	abcdefghijk

Number	Line	Log transformed REML adjusted mean	5% LSD
100	G124	0.2191	abcdefghijk
101	G82	0.2286	abcdefghijk
102	G38	0.2327	abcdefghijk
103	G211	0.247	bcdefghijk
104	G105	0.2499	bcdefghijk
105	G34	0.2534	bcdefghijk
106	G292	0.2605	bcdefghijk
107	G251	0.271	cdefghijk
108	G277	0.2719	cdefghijk
109	G36	0.2754	cdefghijk
110	G142	0.2788	cdefghijk
111	G57	0.282	cdefghijk
112	G249	0.2842	cdefghijk
113	G240	0.2843	cdefghijk
114	G248	0.2846	cdefghijk
115	G26	0.29	cdefghijk
116	G72	0.3007	cdefghijk
117	G241	0.3035	cdefghijk
118	G192	0.3496	efghijk
119	G239	0.36	efghijk
120	G234	0.3656	efghijk
121	G69	0.3722	fghijk
122	G225	0.378	fghijk
123	G92	0.3787	fghijk
124	G6	0.4351	ghijk
125	G288	0.4616	hijk
126	G247	0.4907	ijk
127	G87	0.5392	k
d.f.	126		
5% LSD	0.1693		